ADIPONECTIN IN HEALTH AND DISEASE

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“If we knew what we were doing, it wouldn’t be called research, would it?”

-Albert Einstein

Abstract

The incidence of diseases associated with the metabolic syndrome has rapidly increased in recent years. The most common of these diseases is Type 2 Diabetes. Research into ways of alleviating the pathogenicity of Diabetes is ongoing, and the increase in diagnosis in recent years has motivated scientists to investigate novel risk markers to help predict and prevent Type 2 Diabetes Mellitus and the diseases associated with it.

Adiponectin has become an important molecule in this search. Despite being released from adipose tissue, adiponectin correlates inversely with body fat in humans and animals. It also exhibits important metabolic regulatory functions such as glucose regulation and fatty acid catabolism and has been suggested to have anti-inflammatory properties.

This thesis reviews literature on the adiponectin molecule and aims to explore the complex functioning of this adipokine and its relationship to Cardiovascular Disease (CVD) and Diabetes. The *methodological considerations* chapters focus on pre-analytical and analytical variables that may affect the collection of blood and the measurement of the molecule. We observed the molecule to be very stable. The measurement of both the high molecular weight (HMW) and total adiponectin species were not affected by up to 7 freeze thaw cycles. Furthermore, blood processing times and temperatures did not significantly alter results. Although the R&D systems adiponectin kits do not advise the use of citrated plasma, we validated its use in these kits and although absolute concentrations were lower than with EDTA plasma, they were consistently lower throughout the measured sample set. Two other commercial kits (Mercodia and ALPCO diagnostics) were tested for performance against the R&D systems Enzyme-Linked Immunosorbent Assay (ELISA) kit. Although there were differences between absolute values in each kit, the overall performance of the kits were satisfactory as judged by Bland-Altman plots.

In the first of the two clinical associations chapters, we report, from a prospective study of older British women, no evidence of any association of HMW adiponectin (or its ratio to total adiponectin) with incident vascular events and

suggest that circulating concentrations of adiponectin (and its fractions) may be more strongly aligned to the risk for Diabetes than to vascular events.

The final study investigates the relationship between B-type natriuretic peptide (BNP) and adiponectin in Acute Coronary Syndrome (ACS) patients. Adiponectin and BNP are both known to be positively associated with risk of poor outcome, and with each other, in cross-sectional studies. However, serial changes in these parameters, following ACS, have not previously been measured. In this study, adiponectin and BNP positively correlated at baseline, 7 weeks, and importantly, change over 7 weeks in both parameters was significantly correlated. We reported that increases in plasma adiponectin (rather than absolute levels) after ACS are related to risk of adverse outcome, but that this relationship is not independent of BNP levels. Our results allude to a potential direct or indirect effect of BNP on adiponectin levels, post-ACS; an observation that requires further investigation.

In summary, this thesis has shown adiponectin to be a very stable and robust analyte in plasma or serum, with good reproducibility within persons and broadly between differing assays. Whilst there are clear data linking low adiponectin with incident diabetes, our clinical studies show adiponectin has more complex associations with vascular disease, perhaps mediated in part by a complex interaction with BNP. Further genetic studies are needed to elaborate causal association for this complex molecule.

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Author's Declaration

I declare that I am the author of this thesis and that no part of it has been previously reported in any other thesis. Chapters 5 and 6 draw on results and conclusions from published journal articles, upon which I have been named as a contributing author. All other authors are fully credited where appropriate.

The experimental work on the pre-analytical and analytical variables was carried out by myself in the Division of Cardiovascular and Medical Sciences at the Glasgow Royal Infirmary. Adiponectin analysis on samples in chapters 5 and 6 was carried out by myself (~50% of the work load) or by a technical colleague within our own department. Furthermore, I helped contribute to discussions on the data which helped shape some of the conclusions in the published papers. I also presented one of the papers as part of a national conference. Analysis of all other parameters included in these studies was carried out by other centres of research included in the study grants. Copies of the publications arsing from these studies can be found in the appendix.

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signed: _____

Abreviations

ABCA-1	– ATP Binding Cassette A1
ACC	– Acetyl Co-enzyme A
ACD	– Acid Citrate Dextrose Solution
ACRP30	- Adipocyte Complement Related Protein of 30 kD
ACS	– Acute Coronary Syndrome
ADIPO-R1	– Adiponectin receptor 1
ADIPO-R2	– Adiponectin receptor 2
ADP	– Adenosine diphosphate
AICAR	– Aminoimidazole carboxamide ribonucleotide
ALPCO	– American Laboratory Products Company
AMP	– Adenosine monophosphate
AMPK	– AMP-activated proteína kinase
API	– Activator Protein 1
apMI	– Adipose most abundant gene transcript 1
Apo	– Apolipoprotein
ATP	– Adenosine Triphosphate
BMI	– Body Mass Index
BNP	– Brain Natriuretic Peptide
BRHS	– Bristish Regional Heart Study
BWHHS	– British Woman's Heart and Health Study
CAD	– Coronary Artery Disease
cAMP-PKA	– Cyclic adenosine monophosphate/protein kinase A
C/EBP	– CCAAT/enhancer binding protein
CHD	– Coronary Heart Disease
CI	– Confidence Interval
COOH	– Carboxylic acid
COX-2	– Cyclooxygenase type 2
CPT-1	– Carnitine Palmitoyltransferase 1
CRP	– C-Reactive Protein
CV	– Coefficient of Variation
CVD	– Cardiovascular Disease
DBP	– Diastolic Blood Pressure
EDTA	– Ethylenediaminetetra-Acetic Acid

ELISA	– Enzyme-linked Immunosorbent Assay
ET-1	– Endothelin 1
FABP	– Adipocyte Fatty Acid Binding Protein
FAT	– Fatty Acid Translocase
FATP	– Fatty Acid Transport Protein
gC1q	- Globular domain of adiponectin
GLP	– Good Laboratory Practice
GLUT-4	– Glucose Transporter 4
HDL	– High Density Lipoprotein
HIV	– Human Immunodeficiency Virus
HMW	– High Molecular Weight
HOMA-IR	– Homeostasis Model Assessment – Insulin Resistance
HPFS	– Health Professionals Follow Up Study
ICAM	– Intercellular Adhesion Molecule
I_kB	– Inhibitor Kappa B
IL-1	– Interleukin 1
IL-6	– Interleukin 6
IQR	– Inter-quartile Range
IR	– Insulin Resistance
IS	– Insulin Sensitivity
KDa	– Kilodalton
LDL	– Low Density Lipoprotein
LiHep	– Lithium Heparin
LMW	– Low Molecular Weight
LPL	– Lipoprotein Lipase
MCP-1	– Monocyte Chemoattractant Protein 1
MDD	– Minimum Detectable Dose
MI	– Myocardial Infarction
MMP-9	– Matrix Metalloproteinase 9
MMW	– Middle Molecular Weight
MoAbs	– Monoclonal Antibodies
mRNA	– Messenger Ribonucleic Acid
NF_kB	– Nuclear Factor Kappa B
NH_2	– Amino group
PEPCK	– Phosphoenolpyruvate Carboxykinase

PGJ_2	– Prostaglandin J_2
PPAR	– Peroxisome Proliferator Activated Receptor
PPRE	– Peroxisome Prolierator Response Element
QC	– Quality Control
R&D	– R&D systems supplier
RXR	– Retinoid X Receptor
SBP	– Systolic Blood Pressure
SD	– Standard Deviation
SOP	– Standard Operating Procedure
SR-B1	– Scavenger Receptor Class B1
STAT	– Signal Transducers and Activators of Transcription Protein
TG	– Triglyceride
TNF-α	– Tumor Necrosis Factor Alpha
TZD	– Thiazolidinedione
VCAM	– Vascular Cellular Adhesion Molecule
WAT	– White Adipose Tissue
WHR	– Waist to Hip Ratio

Chapter I: Introduction

1.1 The Adiponectin Molecule

1.1.1 *Background Information to the Molecule*

Adiponectin was originally identified, and given different names, by four independent groups over a decade ago. It was first discovered by Scherer and Lodish (Scherer et al, 1995)) who named it adipocyte complement-related protein of 30kDa (ACRP30), in a subtractive hybridization screen comparing 3T3-L1 adipocytes (cloned cell lines from mouse embryos with the ability to differentiate into cells resembling adipocytes (Panigrahy et al, 2002)) with undifferentiated pre-adipocytes. Spiegelman and colleagues (Hu et al, 1996) then adopted the same approach but they called the protein they isolated, AdipoQ. Human adiponectin, which is 83% the same as the mouse polypeptide, was first cloned by Matsuzawa and colleagues (Maeda et al, 1996) who called it adipose most abundant gene transcript 1 (apM1). Finally Tomita and colleagues (Nakano et al, 1996) employed a very different method by isolating adiponectin from human plasma by utilising its affinity for gelatine, thereby naming it gelatine binding protein. In this thesis I will refer to the molecule as the better known name, adiponectin.

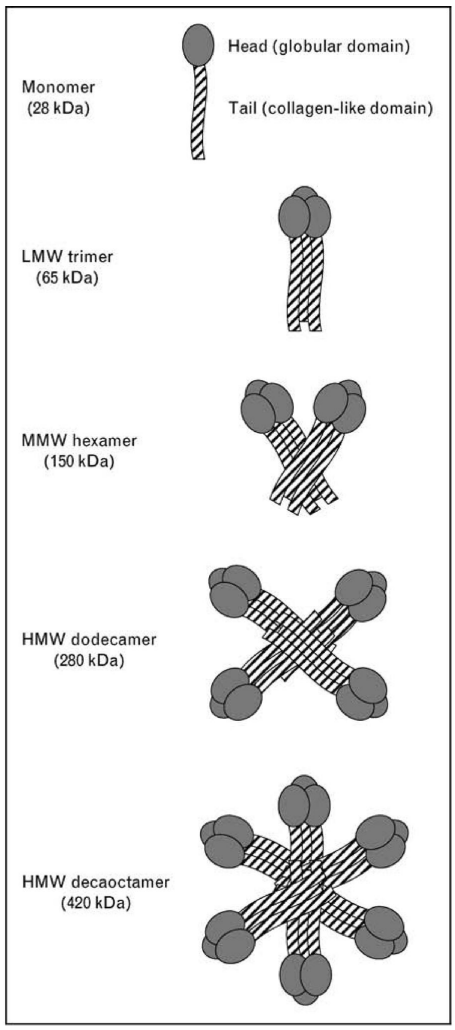
After the first description of the molecule in 1995 there was a gap in literature (Whitehead et al, 2006), and the physiological and pathophysiological importance has only been well documented in recent years. Greater than 4000 publications have implicated this molecule's role in various pathological states (Shetty et al, 2009). The adiponectin gene encodes a secreted protein expressed predominantly in white adipose tissue (WAT) and brown adipose tissue (Kadowaki et al, 2005). It circulates at high levels in human plasma accounting for approximately 0.01% (0.5-30 ug/mL) of all plasma protein in normal individuals (Magkos et al, 2007), ~1000-fold higher than other hormones such as leptin and insulin. Gender has an effect on concentrations of adiponectin, with females having higher levels than males (Xu et al, 2005). It is also well known that adiponectin levels increase with

age, however the cause of this is still unknown (Sattar et al, 2008). Sattar and Nelson (Sattar et al, 2008) suggest rises in age related adiponectin may be as a result of a salvage mechanism against age related illness such as metabolic dysfunction, atherosclerosis and other inflammatory conditions. This will be discussed later in section 1.2.1 of this chapter on how adiponectin relates to cardiovascular disease (CVD) and heart failure.

1.1.2 Structure

The molecule is a 244-amino-acid long adipokine secreted from adipocytes (www.RnDSystems.com). The gene product is a 30kD protein (Scherer et al, 1995), however this is not found in circulation. Adiponectin automatically self-binds to form larger structures and there are different multimeric forms including low molecular weight (LMW) trimers, middle molecular weight (MMW) hexamers, high molecular weight (HMW) oligomeric structures and finally globular adiponectin (gC1q domain) (Figure 1.1 (Magkos et al, 2007)). It has been proposed that this globular fragment is generated by proteolytic cleavage of adiponectin multimers by leukocyte elastase secreted from activated monocytes and/or neutrophils (Kadowaki et al, 2005); however the pathophysiological importance of this cleavage remains to be determined.

Figure 1.1: The Different Multimeric Forms of Adiponectin

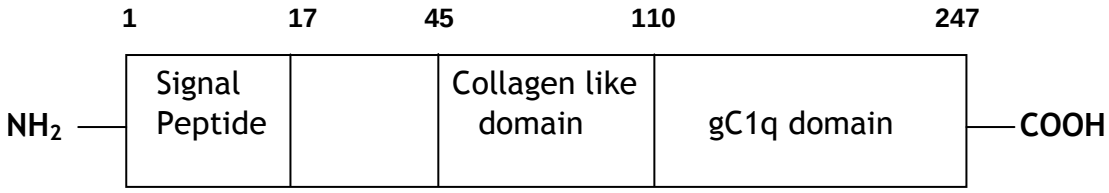


Magkos et al, 2007 (Curr Opin Clin Nutr Metab Care)

1.1.2.1 Monomeric structure

There are four distinct regions of adiponectin (Figure 1.2). The protein starts with a short signal sequence which acts to target the hormone for secretion outside the cell, then it leads into a short region that is variable between species, followed by an amino-acid region that shows similarity with collagenous protein, and finally ending with a globular domain.

Figure 1.2: Schematic Diagram Showing the Protein Structure of Adiponectin



Abbreviations for figure 1.2: COOH, Carboxylic acid; gC1q, Globular domain; NH2, Nitrogen di-hydroxide (amine group).

Takeuchi et al, 2007 (Med Mol Morphol)

1.1.2.2 Higher order structures

Initially, three adiponectin molecules bind together to form a homotrimer. The trimers continue to self-associate and form hexamers or dodecamers (Tilg et al, 2006). Like the plasma concentration, the relative levels of the higher-order structures are sexually dimorphic (Xu et al, 2005; Hug et al, 2004), where females have increased proportions of the high-molecular weight forms. The varying forms have altered biological activity and therefore may also have separate functions. The gC1q domain and the trimeric forms of adiponectin activate AMP Kinase in skeletal muscle and lead to increased fatty acid oxidation and reduction in glucose concentrations (15), whereas the hexameric and full length HMW forms are thought to activate nuclear factor kappa B (NF-κB) pathways (Hug et al, 2004). Conflicting literature on this association will be discussed in detail below in section 1.4.1. The proportion of HMW adiponectin within adipose tissue is higher than in blood plasma (Kadowaki et al, 2005), suggesting regulation at the level of secretion is a mechanism of adiponectin complex distribution. All isoforms of the molecule

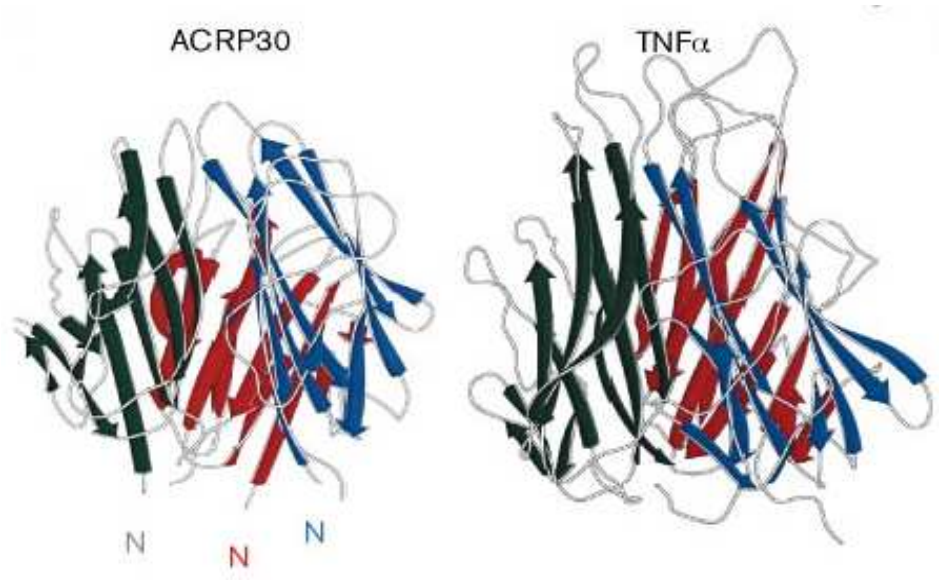
are stable in circulation, do not seem to be able to interconvert and are relatively long-lived (half-life of ~15hrs) (Kadowaki et al, 2005).

1.1.2.3 Homology to Tumour Necrosis Factor alpha (TNFα)

Despite there being unrelated protein sequences, the 3-dimensional structure of the gC1q domain of adiponectin has a striking homology to TNFα, and has been subsequently named as a TNF superfamily member. TNFα is a cytokine involved in systemic inflammation and the acute phase response. It induces the hepatic expression of acute phase proteins. The molecule seems to possess both growth promoting and growth inhibiting properties, as well as mastering a self regulatory function. In spite of the lack of homology at the primary amino acid sequence level, the structural features between TNFα and adiponectin are highly conserved (Scherer et al, 1995) (Figure 1.3 (Schapiro et al, 1998)). Both proteins form bell- shaped homotrimeric oligomers, and the evolutionary relationship between adiponectin and TNF family proteins suggests that the human adiponectin receptor may also be a member of the TNF receptor superfamily.

Figure 1.3: Crystal Structure of the Globular Domain of Adiponectin (ACRP30 gC1q domain) and comparison with the TNF*a* trimer

The amino terminus of each ACRP30 protomer is labelled. The collagen stalks would protrude from these amino termini. The three-fold axis is non-crystallographic. The three colours highlight the fact that both proteins form homo-trimeric oligomers.



Abbreviations for figure 1.3: ACRP30, Adipocyte Complement-Related Protein of 30kDa; TNFα, Tumour Necrosis Factor Alpha.

Schapiro et al, 1998 (Current Biology)

1.1.3 Receptors and Signaling

Two receptors have been identified that bind adiponectin: Adipo R1 and Adipo R2. AdipoR1 was first identified when encoding cDNA was isolated from human skeletal cDNA library by screening for adiponectin binding (Takeuchi et al, 2007). The AdipoR2 was identified later due to its striking homology to AdipoR1. Both are surface membrane proteins (Takeuchi et al, 2007) and have homology to G protein- coupled receptors. The receptors have different affinities to the various molecular forms of adiponectin. AdipoR1 and AdipoR2 are found in liver, muscle and adipose tissue in humans; however AdipoR1 is predominantly expressed in skeletal muscle whereas AdipoR2 is more predominant in the liver (Takeuchi et al, 2007). AdipoR1 is a high-affinity receptor for globular adiponectin as well as having a lower affinity for full length adiponectin. In contrast the AdipoR2 receptor has an equal intermediate affinity for globular and full length HMW adiponectin (Takeuchi et al, 2007). The receptors affect a very important cellular metabolic rate control point, by targeting AMP-activated protein Kinase (AMPK), downstream. AMPK is a stress induced kinase that is activated in response to depleting Adenosine triphosphate (ATP) or increasing Adenosine monophosphate (AMP) levels. AMPK activates ATP and generates catabolic processes such as fatty acid breakdown and glycolysis and shuts down ATP-consuming processes such as lipogenesis (Shetty et al, 2009). Expression of these receptors is correlated with insulin levels (Kadowaki et al, 2005). A review of studies involving the adiponectin receptors suggests that they may have an important role in adiponectin physiology. Key findings suggest that changes in expression of AdipoR isoforms in skeletal muscle (rather than total circulating adiponectin concentrations) may be of physiological importance (Vu et al, 2007), although this may reflect a form of “adiponectin resistance” particularly in less healthy individuals.

More recently, by means of expression cloning, T-cadherin has been recognized as an adiponectin receptor on vascular endothelial cells and smooth muscle (Takeuchi et al, 2007). The expression of this cadherin molecule is known to be correlated with atherosclerosis (Tilg et al, 2006), the pathogenesis of which adiponectin is thought to have an effect on. T-cadherin and adiponectin also both show protection against vascular endothelial cell

apoptosis which means that they may have overlapping biological properties in injured endothelial cells (Tilg et al, 2006). The receptor is a glycosylphosphatidylinositol-anchored[1] extra-cellular protein that binds hexameric and HMW adiponectin but not trimeric or globular species (Hug et al, 2004). Interestingly the amino acid sequence of T-cadherin has been well conserved through evolution, suggesting that it may indeed play an important role in higher order vertebrates (Tilg et al, 2006). T-cadherin lacks a cytoplasmic domain, and therefore it is believed that it does not act as a cell-cell adhesion molecule and is not restricted to cell-cell boundaries but is rather found over the entire surface membrane (Tilg et al, 2006). The molecular mechanisms of any transmission of the adiponectin signal into the cytoplasm and nucleus remain unclear. Hug et al (Takeuchi et al, 2007) reported that only eukaryotically expressed adiponectin bound to T-cadherin, implying that there was some post translational modification of the molecule critical for binding. Although the understanding of the roles and regulation of these modifications is incomplete, it is clear they contribute to adiponectin multimerisation and function. The cysteine residue situated in the amino terminal variable domain of adiponectin is involved in disulphide bond formation, essential for the oligomerisation process (Whitehead et al, 2006). Other post-translational modifications include hydroxylation of conserved proline and lysine residues within the variable and collagenous domain, and these lysine residues are further modified by the addition of a glucosylgalactosyl group. The structural significance of these modifications has not been reported but with a mutation in four of the five genes encoding the lysine residues, a significant decrease in the insulin-sensitising action of adiponectin is seen (Nakano et al, 1996).

Hug's study (Hug et al, 2004) also suggests that because of its extracellular nature, T-cadherin may also act as a co-receptor for as yet unidentified signaling receptors, through which adiponectin transmits metabolic signals. It is also

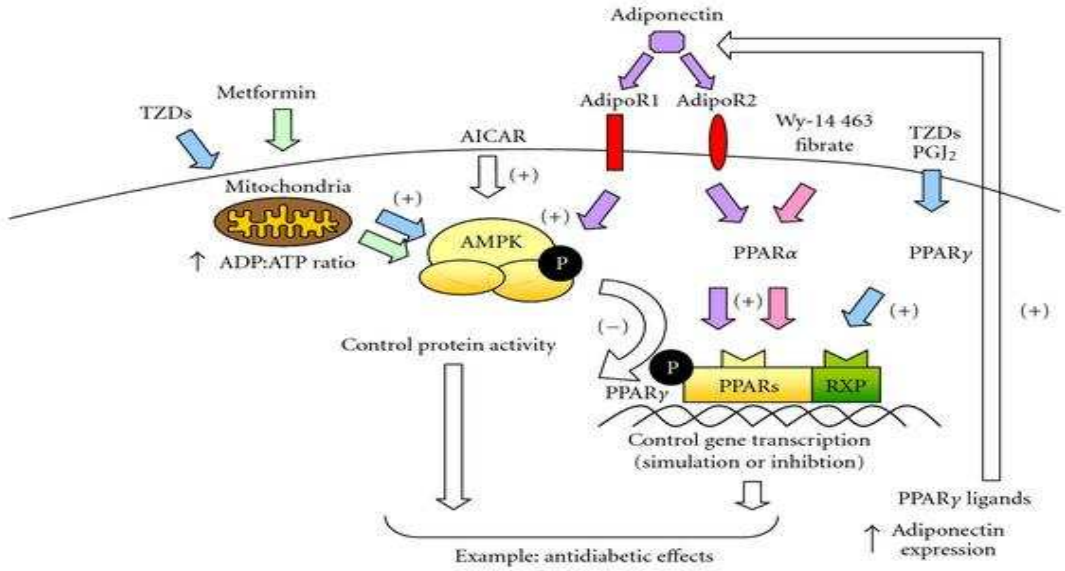
[1] Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are a functionally and structurally diverse family of post-translationally modified membrane proteins found mostly in the outer leaflet of the plasma membrane in a variety of eukaryotic cells. Although the general role of GPI-APs remains unclear, they have attracted attention because they act as enzymes and receptors in cell adhesion, differentiation, and host-pathogen interactions (Elortza et al, 2003).

possible however that T-cadherin may be acting as a non-signalling decoy receptor of adiponectin. More studies of this nature are required to unravel the mystery of the T-cadherin/adiponectin signal pathway.

Adiponectin is also known to be involved in is the regulation of peroxisome proliferator-activated receptor (PPAR) activity (Kadowaki et al, 2005). PPARs play an important role in cellular functioning including lipid metabolism, cell proliferation, differentiation, adipogenesis and inflammatory signalling (Han et al, 2008). There exist three different isomers of the PPAR (α, ß,ɣ), each with its own ligand activators and each being involved in different processes within the cell, ranging from transcription mediation to fatty acid storage. For example a PPARα agonist increases the expression of adiponectin receptors in WAT and a PPARɣ agonist increases the amount of high molecular weight multimers compared to total adiponectin (Tsuchida et al, 2005). Treatment of obese animals by specific PPAR ß agonists causes metabolic parameters to normalise and results in the reduction of adiposity (Tenenbaum et al, 2005) and hence may prevent the development of obesity (Yamauchi et al, 2002). When adiponectin is secreted from WAT it binds to its receptor (AdipoR1 or AdipoR2). With this binding it activates AMPK and PPARα (figure 1.4 (Dupont et al, 2008)). Ligand bound PPAR forms a heterodimeric complex with the retinoid X receptor (RXR) which is capable of binding to specific response elements, termed peroxisome proliferator response elements (PPRE) (Figure 1.5 (Gervois et al, 2007)). This interaction in the promoter region of target genes allows the PPAR to regulate gene expression, in particular adiponectin gene expression. Therefore, adiponectin is, to some extent self-regulatory through PPAR. PPARs may repress gene transcription by a mechanism that is independent of DNA binding (left of figure 1.5).This mechanism includes interference by PPARs with other transcription factor pathways such as those involving NFκB, signal transducers and activators of transcription protein (STAT), CCAAT/enhancer binding protein (C/EBP), and activator protein-1 (AP-1), which modulate the expression of genes involved in inflammation pathways. In the liver, full length adiponectin activates AMPK, thereby reducing the production of molecules involved in gluconeogenesis and increasing phosphorylation of acetyl coenzyme-A carboxylase (ACC) as well as

fatty acid oxidation. In the skeletal muscle both globular and full length adiponectin bind to the receptors to activate AMPK and stimulate phosphorylation of ACC, fatty-acid oxidation and glucose uptake (Kadowaki et al, 2005).These cascades both result in decreased triglyceride content and increased insulin sensitivity in the cells (Figure 1.6 (Kadowaki et al, 2005)).

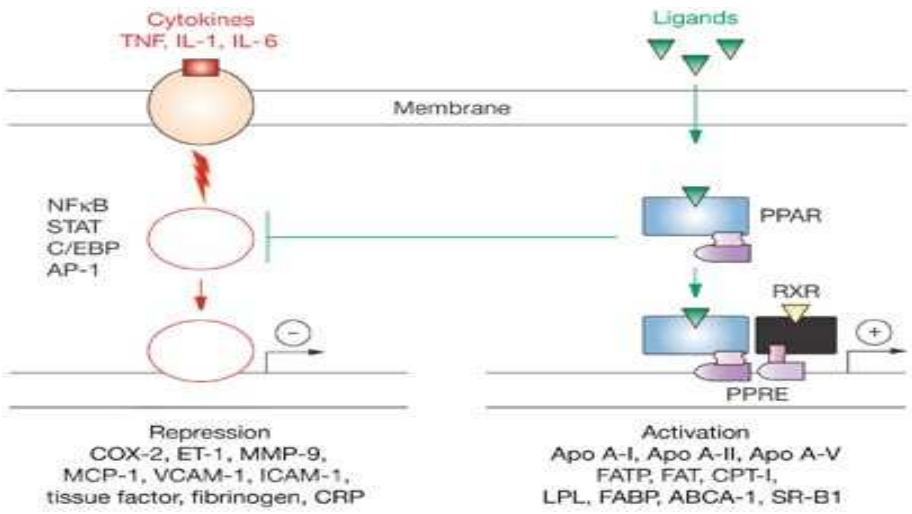
Figure 1.4: Diagram Showing the Activation of AMPK and PPAR by Binding of Adiponectin to Its Receptors.



Abbreviations for figure 1.4: ADP, adenosine diphosphate; ATP, adenosine triphosphate; TZD, Thiazolidinediones; AICAR, aminoimidazole carboxamide ribonucleotide; PGJ_2, Prostaglandin J2

Dupont et al, 2008 (PPAR Research)

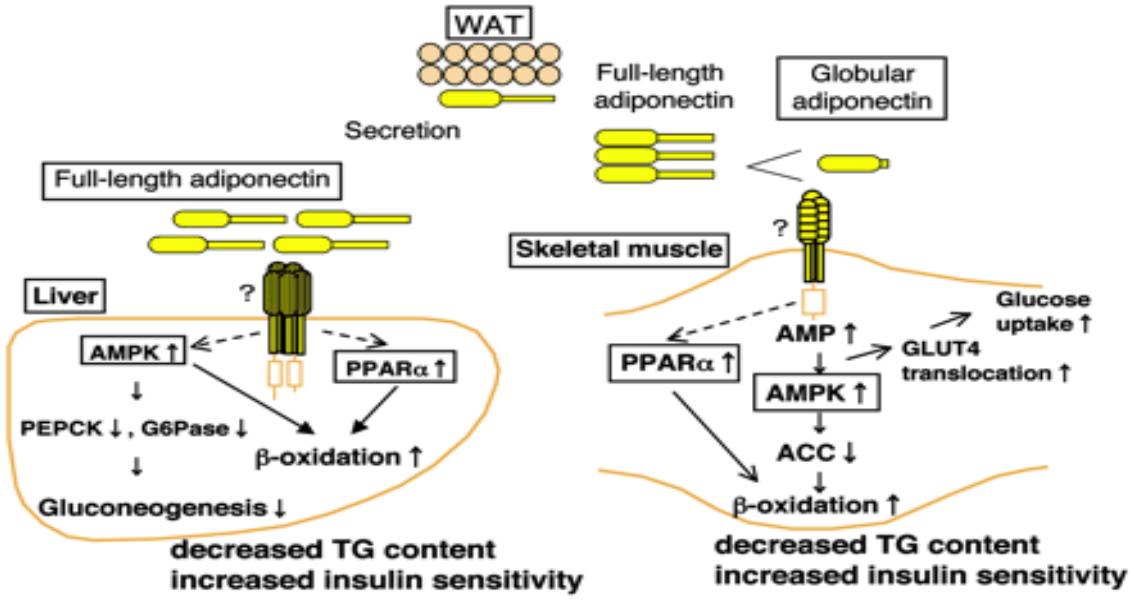
Figure 1.5: Diagram Showing the Methods of Repression by the Ligand Bound PPAR and Activation by the PPAR-RXR Heterodimeric Complex.



Abbreviations for figure 1.5: ABCA-1, ATP-binding cassette A1; Apo, apolipoprotein; COX-2, cyclooxygenase type 2; CPT-I, carnitine palmitoyltransferase I; CRP, C-reactive protein; ET-1, endothelin 1; FABP, adipocyte fatty acid binding protein; FAT, fatty acid translocase; FATP, fatty acid transport protein; IL-1, interleukin 1; LPL, lipoprotein lipase; MCP-1, monocyte chemoattractant protein 1; MMP-9, matrix metalloproteinase 9; SR-B1, scavenger receptor class B1; STAT, signal transducer and activator of transcription.

Gervois et al, 2007 (Nat Clin Pract Endocrinol Metab)

Figure 1.6: A Depiction of the Role of Adiponectin in Activating AMPK and PPARα



Abbreviations for figure 1.6: PEPCK, Phosphoenolpyruvate carboxykinase; GLUT4, glucose transporter 4; TG, Triglyceride.

Kadowaki et al, 2005 (Endocr Rev)

1.1.4 Function

Adiponectin has a number of diverse roles within the body incorporating modulation of a number of metabolic processes. Despite being released from adipose tissue, adiponectin correlates inversely with body fat in humans (Cnop et al, 2003) and animals (Freubis et al, 2001). It also exhibits important metabolic regulatory functions such as glucose regulation and fatty acid catabolism and has been suggested to have anti-inflammatory properties (24).

1.1.4.1 Adiponectin function in *in vitro* and animal studies

In 2001, Freubis et al. showed the apparent insulin sensitizing effects of adiponectin. Mice were fed either a high fat test meal or were injected with fat emulsion, which dramatically increased the amounts of free fatty acid levels. By treatment with protease-generated globular adiponectin, the elevated levels of free fatty acids were significantly lowered. This effect was caused in part by the increase in fatty acid oxidation in the muscle. Kadowaki and Yamauchi (Kadowaki et al, 2005) also verified the direct insulin-sensitizing effect of adiponectin *in vivo* by using an insulin resistant lipoatrophic diabetic mouse model that displayed both adiponectin and leptin[2] deficiency. The test animals were then given a physiological dose of recombinant adiponectin and insulin resistance was significantly reduced. Moreover, Scherer's group reported that an acute increase in circulating adiponectin levels triggers a brief decrease in basal glucose levels by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in wild type and type 2 diabetic mice (Berg et al, 2001). These findings are consistent with the proposal that adiponectin sensitises the body to insulin, and perhaps explains higher expression in the lean: adiponectin expression in adipose tissue may control glycaemia and fatty acid levels in normal physiological circumstances. Yamauchi et al then showed that adiponectin increases AMPK and ACC phosphorylation

[2] Leptin is synthesised and secreted by adipose tissue and acts on the brain and peripheral organs to regulate appetite and energy balance within our body

in muscle, by treating mice with recombinant adiponectin (Yamauchi et al, 2002).

Their findings showed that both globular and full length adiponectin increased AMPK phosphorylation in vivo in a dose dependent manner, with a 2-fold peak at 5 minutes, returning to baseline value after 60 minutes. To determine whether the AMPK pathway was required for adiponectin effect in muscle cells, Yamauchi and colleagues used a catalytically inactive AMPK to demonstrate that activation of AMPK is necessary for adiponectin-induced stimulation of ACC phosphorylation, fatty acid oxidation, glucose uptake and lactate production in muscle cells. This experiment links adiponectin to weight limitation.

1.1.4.2 Adiponectin and its role in inflammation

Adiponectin may act as a modulator for the endothelial inflammatory response by targeting the NF-κB pathway. Members of this family of nuclear factors are responsible for regulating over 150 target genes, including the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules. Because of this, NF-κB has often been called a central mediator of the human immune response.
Adiponectin has been reported to act as both an agonist and an antagonist for the NF-κB pathway. Adiponectin may promote (Tsao et al, 2002; Hug et al, 2004) **or** inhibit (Kadowaki et al, 2005; Ouchi et al, 2000) NF-κB signalling, probably in a context specific manner. This may consequently lead to up regulation or down regulation of the inflammatory response in target cells depending on the context. Hexameric and larger forms of adiponectin (but not the trimeric or globular species) are thought to activate the NF-κB pathway (Tsao et al, 2002; Hug et al, 2004), in a manner dependent upon phosphorylation and degradation of IκB-α. Although the significance of this activity is unclear, the hexameric and HMW isoforms of adiponectin do inhibit apoptosis of endothelial cells (Kobayashi et al, 2004) and NF-κB has well described antiapoptotic activities (Wang et al, 1998). This suggests oligomerisation may have an important role to play in the molecule's biological activity.

On the other hand, adiponectin was shown to inhibit TNF-α-induced nuclear factor-κB activation through the inhibition of IκB phosphorylation (Ouchi et al, 2000) (Figure 1.7 (Tilg et al, 2006)). Through this suppression adiponectin strongly inhibits the expression of adhesion molecules including intracellular adhesion molecule-1 (ICAM), vascular cellular adhesion molecule-1 (VCAM), and E-selectin (Kadowaki et al, 2005). Suppression of nuclear factor-κB by adiponectin might also be a major molecular mechanism for the inhibition of monocyte adhesion to endothelial cells, thus preventing atherogenesis as an anti-inflammatory factor.

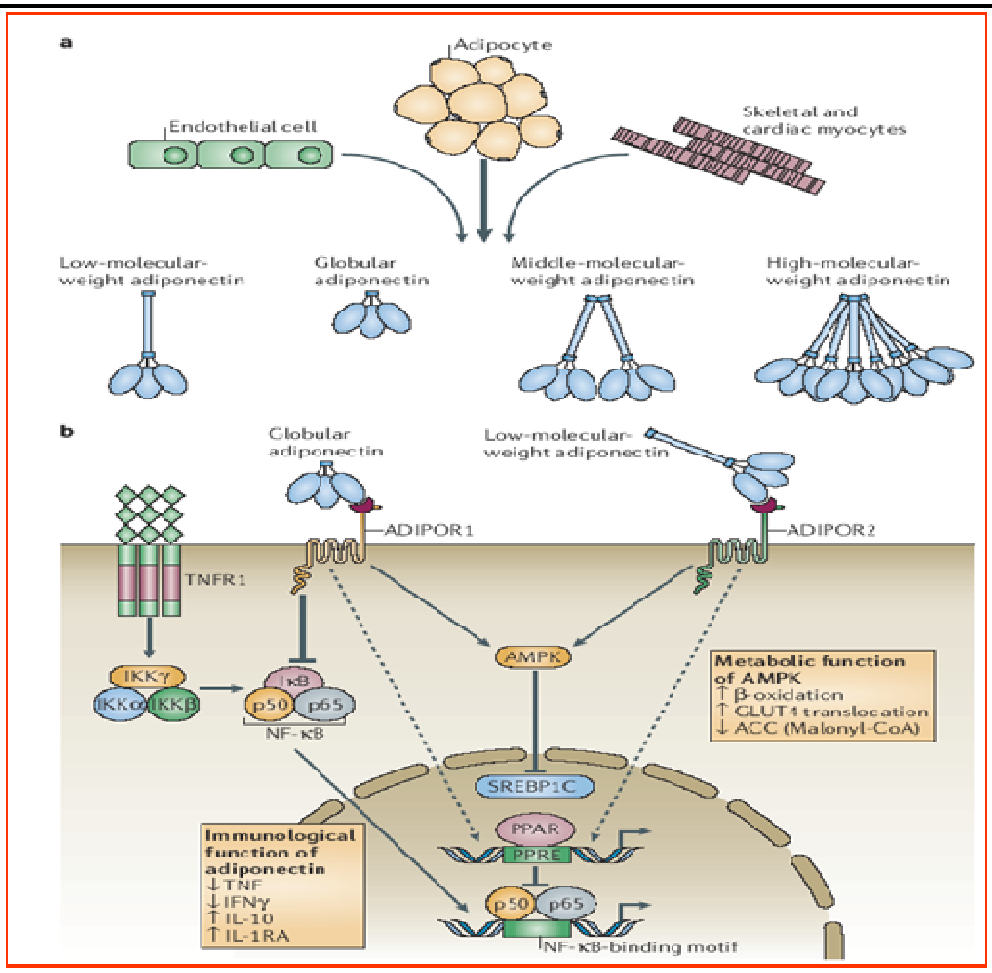
Adiponectin is thought to dampen the early phases of macrophage inflammatory responses, acting to inhibit the growth of myelomonocytic progenitor cells (Yokota et al, 2000) and decrease the ability of mature macrophages to respond to activation (Gervois et al, 2007). Adiponectin also inhibits the expression of the scavenger receptor class A-1 of macrophages, resulting in markedly decreased uptake of oxidized low-density lipoprotein (LDL) by macrophages and inhibition of foam cell formation. This is vital in fighting the onset of atherosclerosis. Normal LDL in plasma is not oxidized. Oxidation of LDL is believed to contribute to the development of atherosclerosis (Yokota et al, 2000), a condition where the walls of the arteries are damaged and narrowed by deposits of plaque (cholesterol and other fatty substances, calcium, fibrin, and cellular wastes), eventually blocking off the flow of blood. Macrophage cells preferentially take up oxidized LDL, become loaded with lipids, and convert into "foam cells" (Aviram et al, 1996) (Figure 1.8 (Chang et al, 1994)). When large numbers of foam cells accumulate in the artery they form a plaque. Stenosis (the narrowing of the artery) occurs with the proliferation of foam cells. The lipid laden plaques become unstable and eventually may rupture resulting in Myocardial Infarction. In addition, in smooth muscle cells, adiponectin attenuated DNA synthesis induced by growth factors (Arita et al, 2002). Some important roles adiponectin plays in inflammation are summarised in figure 1.9 (Iqbal O, 2007).

Okamoto *et al* used apoE deficient mice to demonstrate that an increase of plasma adiponectin suppressed the progression of atherosclerotic lesions (Okamoto et al, 2002). They injected a replication- defective adenovirus

vector containing full length adiponectin or ß galactosidase gene in the tail vein of male apoE-/- mice. These mice develop vascular lesions similar to human atherosclerosis (Plump et al, 1992). After 14 days atherosclerotic lesion areas in the full length adiponectin treated mice were reduced by 30% compared with the ß galactosidase gene treated mice. They also reported a decrease in the m-RNA levels of VCAM-1 and TNFα. These findings suggest that plasma adiponectin may protect endothelial cells from hyper-cholesterolemia-vascular injury, and reduce the uptake of LDL into foam cells.

Figure 1.7: Illustrates Adiponectin's Role in the Inhibition of the NF-κB Pathway.

Note that adiponectin acts in the inhibition of the TNF-α induced NF-κB pathway (b).

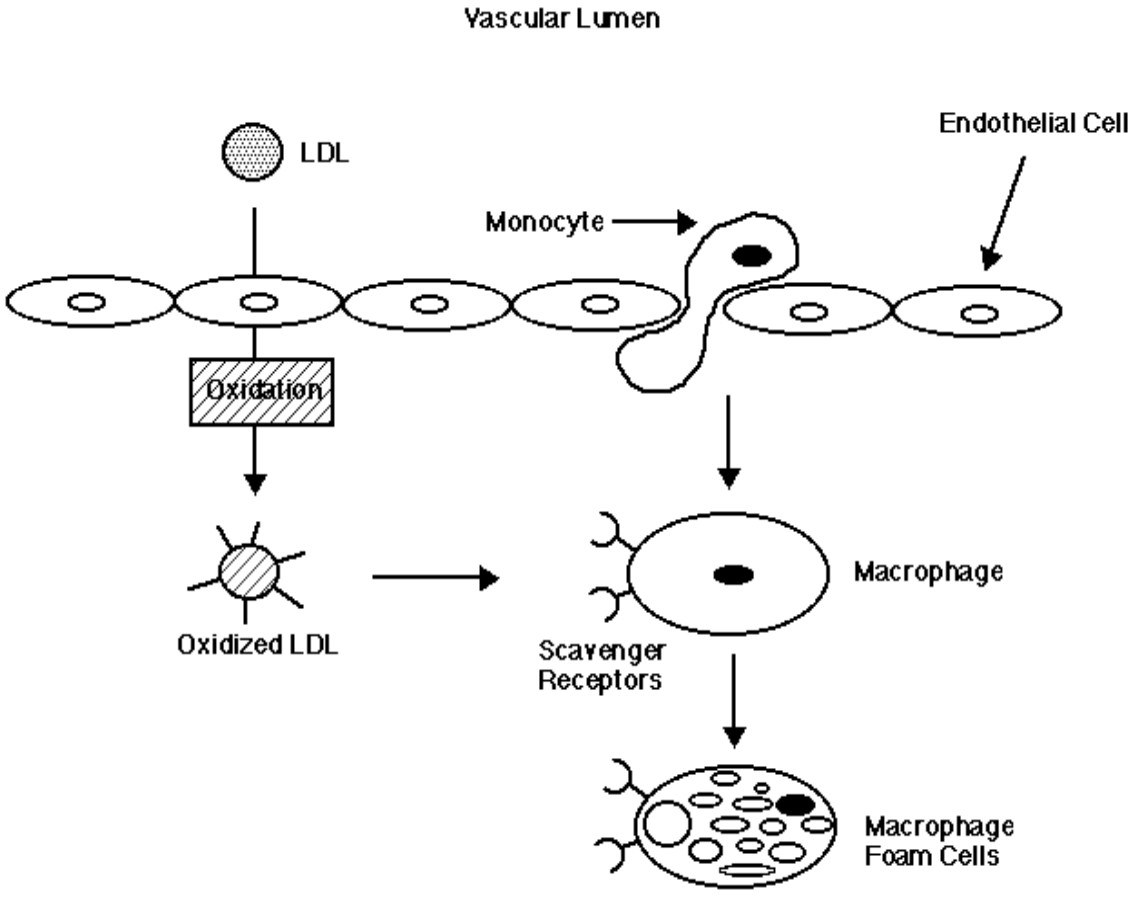


Abbreviations for figure 1.7: AdipoR1, Adiponectin receptor 1; AdipoR2, Adiponectin receptor 2; TNFR1, Tumour necrosis factor receptor 1; AMPK, amp-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; PPRE, Peroxisome Proliferator Response Element; IκB, IkappaB kinase; IFNγ, Interferon y; IL-10, Interleukin 10; IL-1RA, Interleukin 1 receptor antagonist.

Tilg et al, 2006 (Nat Rev Immunol)

Figure 1.8: Diagram Showing Oxidation of LDL and Foam Cell Formation

Monocytes migrate from the vascular lumen to the intima where they differentiate into macrophages. The scavenger receptors on the surface of macrophages take up modified LDLs, forming macrophage foam cells.

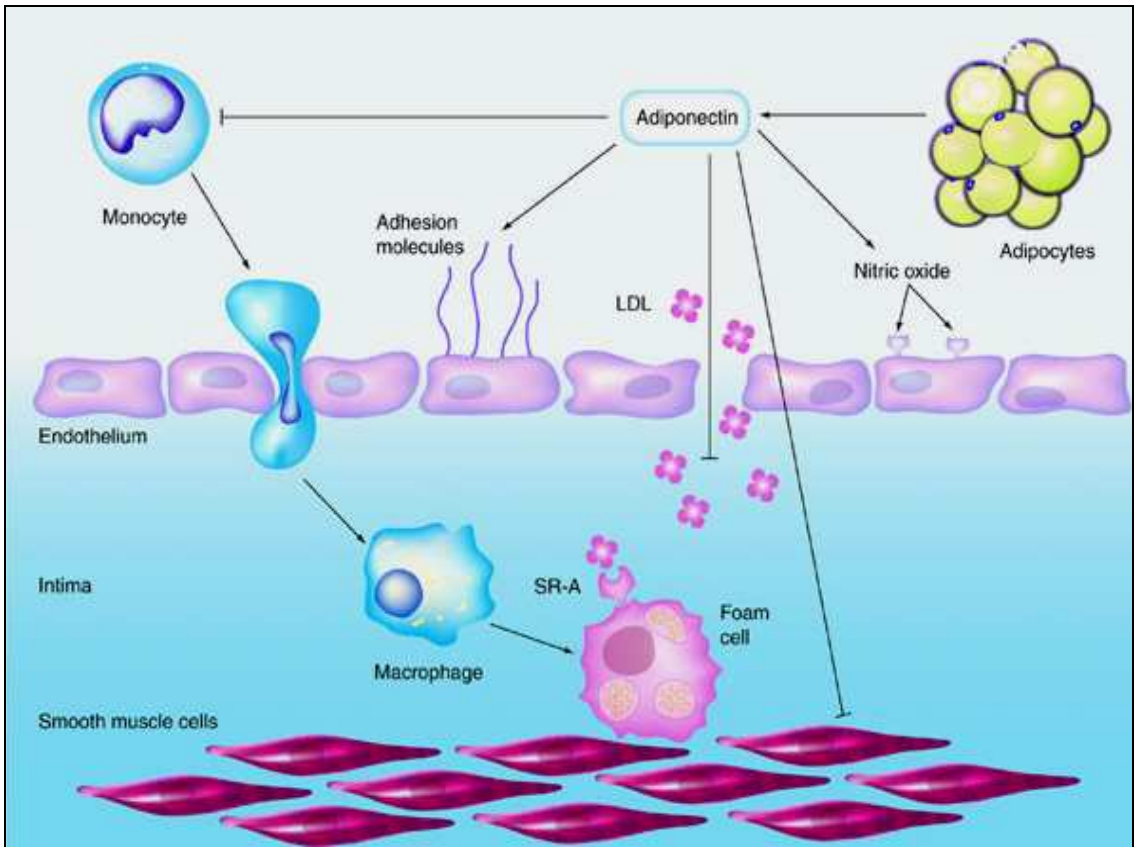


Abbreviations for figure 1.8: LDL, Low density Lipoprotein

Chang et al, 1994 (Somat Cell Mol Genet)

Figure 1.9: Summarising the Anti-Inflammatory Role of Adiponectin

Adiponectin acts in the inhibition of monocyte adhesion to endothelial cells and prevents the oxidation of LDL, thus preventing atherogenesis as an anti-inflammatory factor.



Abbreviations for figure 1.9: LDL, Low density lipoprotein; SR-A, Scavenger receptor A

Iqbal O, 2007 (Personalized Medicine)

1.1.4.3 Associations of Adiponectin with other Risk Factors in Humans

Adiponectin is a very complex hormone and exhibits relationships with a number of molecules, and performs a number of diverse roles within our body. A few of these processes have been demonstrated by means of in vitro and animal studies, but how does the molecule function in humans?
Since the discovery of the insulin-sensitising action of adiponectin in 2001 (Kadowaki et al, 2006), there has been great interest in the molecule. Substantial research has been undertaken with the aim to develop a recombinant form of the protein for therapeutic purposes. Clinical studies have revealed negative associations with body mass index (BMI), waist to hip ratio (WHR), Insulin, glucose, HOMA-IR, C-reactive protein (CRP) levels and triglyceride levels while exhibiting positive associations with High density lipoprotein (HDL)-Cholesterol and age (Table 1.1 (Sattar et al, 2008). Many studies (Ouchi et al, 2007; Pischon et al, 2004; Rathman et al, 2000; Wannamethee et al, 2007; Zhuo et al, 2009)) have found similar associations. Hu *et a*l first showed that adiponectin may be linked to human obesity (Hu et al, 1996) by using northern blots to show that adiponectin expression was reduced in adipose tissues of obese mice and humans (Lu et al, 2008). A report of an adiponectin immunoassay stimulated activity in the human clinical research field (Arita et al, 1999). Using this assay Arita *et al* demonstrated that plasma adiponectin levels were higher in women than in men (Lau et al, 2008) and in non-obese than obese subjects. These reports have subsequently been confirmed by many other studies (Kadowaki et al, 2006; Whitehead et al, 2005; Hulstrom et al, 2008). Despite adiponectin being secreted by adipocytes, circulating levels decrease with obesity. Research originally proposed that mechanisms associated with insulin resistance, such as inflammation, inhibited the expression of adiponectin in the adipose tissue (Lu et al, 2008). Cytokines released in inflammation, which are known to rise in obesity, may play a role in the suppression of adiponectin. Recent studies in human subjects involving the treatment with PPARγ agonists revealed that the plasma adiponectin levels increase 2-fold despite significant body weight gain (Lu et al, 2008). This means that adiponectin levels are increasing independently of weight and rather due to improvement in adipose-specific

insulin sensitivity. Therefore it is now thought adiponectin gene expression may increase irrespective of changes in adiposity but rather due to adipose tissue specific insulin sensitivity (Lu et al, 2008).In line with this Vozarova *et al* showed that hypo-adiponectinemia is not the cause of obesity but rather a result of obesity induced insulin resistance in the adipose tissue (Vozarova et al, 2002). In this study low plasma adiponectin concentrations did not predict future weight gain in humans. This result was also seen in animal models (Pelleymounter et al, 1995). Therefore it may be leptin, rather than adiponectin, that is the hormone primarily responsible for weight regulation. Paradoxically to adiponectin, serum levels of leptin increase with weight gain.

Considering the metabolic effects of adiponectin, it is fair to propose that the lower levels of adiponectin seen in obese subjects is a direct result of the obesity and adipose tissue specific insulin resistance and that these lower levels of adiponectin mediate the state of insulin resistance and metabolic outcomes in other peripheral tissues. Consistent with the known associations of adiponectin with obesity and diabetes, multiple observations now support the molecule as a potent antidiabetic hormone. In a number of prospective diverse population studies, adiponectin consistently predicts lower risk for Type 2 Diabetes (Kadowaki et al, 2005; Sattar et al, 2008; Sattar et al, 2008) and as predicted lower adiponectin levels were also reported in many insulin resistance-related clinical conditions such as polycystic ovary syndrome, non-alcoholic fatty liver disease, lipodystrophy and human immunodeficiency virus (HIV) patients (Lu et al, 2008).

Collectively these observations would suggest that adiponectin is predominantly beneficial, leading us to the assumption it may also display protective effects in vascular tissue. In 2004 Pischon *et al* reported an outcome in apparent protection against myocardial infarction (MI) (Pischon et al, 2004). The initial excitement of these findings later dissipated as increasing numbers of published journal articles appeared to quash the idea, and the latest literature now shows higher levels of circulating adiponectin actually correlate positively to coronary heart disease (CHD) and all cause mortality, rather than to apparent protection against CHD (Laughlin et al, 2007). Some ideas as to the reason for this will be discussed in more detail

later on. The relationship between adiponectin and blood pressure seemed to be more difficult to demonstrate in human studies, probably due to the interference from other metabolic confounding factors related to insulin resistance. But results lean towards there being a higher reported blood pressure with lower plasma adiponectin levels (Lu et al, 2008).

Although a large number of studies have researched the complex mechanisms of this molecule and its associations with other risk markers, larger studies with increased sample numbers are needed to elucidate these potential links.

Table 1.1: Correlation of High Molecular Weight Adiponectin and Total Adiponectin with Measures of Adiposity, Insulin, Glucose and Lipid Levels in British Women Aged 60-79 years.

N = 500

	Spearman's Rank correlation coefficient with HMW adiponectin	p	Spearman's Rank correlation coefficient with total adiponectin	p
HMW or total adiponectin	0.75	< 0.001	0.75	< 0.001
Age	0.08	0.07	0.13	0.004
BMI	-0.12	0.008	-0.20	< 0.001
Waist-hip ratio	-0.26	< 0.001	-0.32	< 0.001
Insulin	-0.35	< 0.001	-0.41	< 0.001
Glucose	-0.11	0.01	-0.20	< 0.001
HOMA-IR	-0.30	< 0.001	-0.38	< 0.001
CRP	-0.17	0.002	-0.20	< 0.001
Total chol.	0.00	0.97	-0.02	0.51
LDL-C	0.01	0.84	-0.01	0.63
HDL-C	0.37	< 0.001	0.44	< 0.001
Triglycerides	-0.34	< 0.001	-0.42	< 0.001
SBP	-0.06	0.20	-0.05	0.36
DBP	0.05	0.31	0.01	0.62

Abbreviations for table 1.1: BMI, body mass index; CRP, C-reactive protein; HOMA-IR, Homeostasis model assessment insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure

Sattar et al, 2008 (J Clin Endocrinol Metab)

1.2 The Associations of Adiponectin with Incident Diabetes Mellitus, Vascular Disease and Mortality

The prevalence of obesity has increased dramatically in recent years, and it is commonly associated with Type 2 Diabetes Mellitus, coronary artery disease (CAD) and Hypertension, collectively known as the metabolic syndrome (Kadowaki et al, 2006). Adipose tissue has been targeted as the prime focus for biomedical research associated with obesity and obesity related diseases (Vozarova et al, 2002). It is now considered to be a genuine endocrine tissue which has the primary role of secretion of molecules into the blood; TNFα, Inter-Leukin 6 (IL-6), leptin, adiponectin, resistin, visfatin and omentin among others, which influence the physiology of organs or tissues at a distant site. Understanding the biology of adipose tissue and the rapidly growing list of adipokines secreted from it provides new insights into normal physiological regulation, as well as the pathogenesis and treatment of obesity, diabetes and disorders of lipid metabolism and the cardiovascular system (Ahima et al, 2008).

1.2.1 Associations with Diabetes Mellitus

Type 2 diabetes is very common in obese individuals. It is a disorder whereby the body still produces insulin but does not produce enough insulin or does not utilise the insulin that is produced correctly (insulin resistance (IR)), or a combination of these. The prevalence of the disease worldwide is reaching epidemic proportions (Smith et al, 2003) and is said to be fuelled by the adoption of a more “westernised” lifestyle incorporating high fat foods and including little exercise. IR is a key feature of the metabolic syndrome and the diseases associated with it. It is now generally accepted to be the primary metabolic defect of Type 2 Diabetes Mellitus (Smith et al, 2003). IR is defined as a state that requires more insulin to obtain the biological effects achieved by a lower amount of insulin in the normal state (Kadowaki et al, 2006).
In contrast to other adipokines, circulating levels of adiponectin correlate inversely with body fat and IR in humans (Whitehead et al, 2006) and rodent models (Kadowaki et al, 2006). Adiponectin is known to increase fatty acid oxidation in the blood, thus improving insulin sensitivity in tissues and

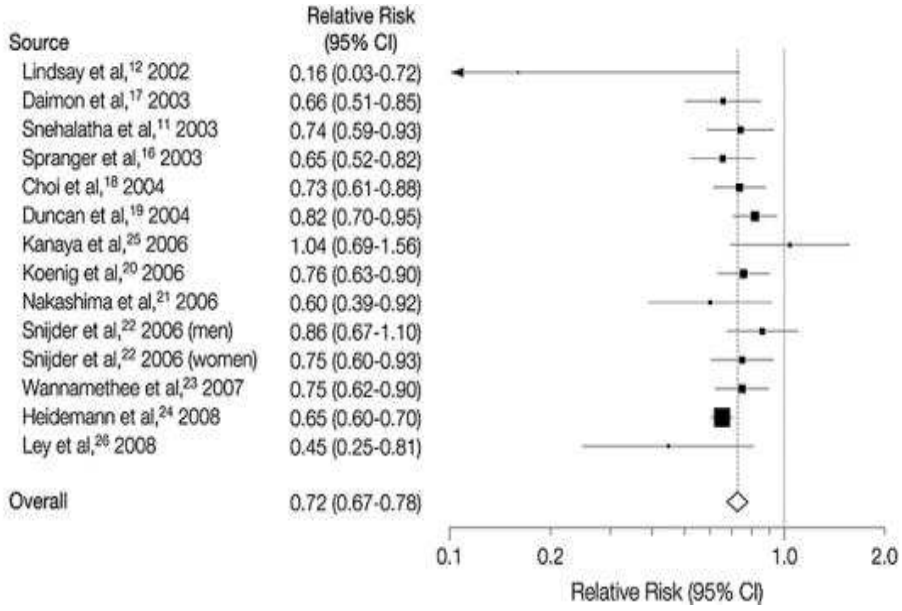
therefore a reduced circulating adiponectin level in the blood may result in insulin resistance. It has been reported that blood concentrations of adiponectin fall from 20-50% in humans and mice respectively with the administration of insulin, thus implying that the effect of insulin to lower adiponectin levels may involve inhibition of adipocyte secretion. Plasma adiponectin levels have also been shown to be decreased in an obese rhesus monkey model that frequently develops Type 2 Diabetes (Kadowaki et al, 2006). Many prospective studies (Yatagai et al, 2003; Daimon et al, 2003; Spranger et al, 2003; Jalovaara et al, 2008) have shown that lower adiponectin levels are associated with a higher incidence of diabetes in humans and more importantly the decrease in plasma adiponectin levels was in parallel with the decrease in insulin sensitivity (Kadowaki et al, 2006). This finding is further supported by a recent meta analysis of 13 prospective studies, which found that higher adiponectin levels were monotonically associated with a lower risk of Type 2 Diabetes across diverse populations (Li et al, 2009) (Figure 1.10 (Li et al, 2009)).

Studies (Yu et al, 2002; Miyazaki et al, 2004) have shown that in type 2 diabetic patients, treatment with medications incorporating thiazolidinedione (TZD) enhances insulin action on peripheral glucose disposal (Miyazaki et al, 2004), reduces hepatic fat content (Yatagai et al, 2003) and decreases endogenous glucose production (Yu et al, 2002). Such medications are also known as glitazones, and are regarded as insulin sensitizing agents. These medications are used in patients with diabetes mellitus where lifestyle measures have proved insufficient (Krentz et al, 2006). Plasma adiponectin increases substantially in response to glitazone treatment in healthy, obese and Type 2 diabetic subjects (Yu et al, 2002; Hulstrom et al, 2008; Miyazaki et al, 2004). The insulin sensitizing effects resulting from treatment with glitazones are thought to be regulated through PPAR-ɣ (Yu et al, 2002) (Figure 1.11 (Kumar CKA, 2008)). Glitazone treatment results in decreased insulin resistance, modified adipocyte differentiation, decrease in leptin levels, a fall in certain interleukin levels, and a rise in adiponectin levels (Wang et al, 2003; Otto et al, 2006). Although it is reasonable to assume that the insulin-sensitising and antidiabetic effect of TZD is mediated in part by up regulation of adiponectin levels, in fact very few studies (Yu et al, 2002; Miyazaki et al, 2004; Pajvani et al, 2004; Phillips et al, 2003; Boden et al, 2003) to date have

reported an association between increases in adiponectin levels and improvements in peripheral and hepatic insulin sensitivity in response to treatment with TZD medications. In one of the above mentioned studies, Pajvani et al postulates that it may be down to the complex distribution of the adiponectin molecule rather than its absolute amount that correlates to TZD-mediated improvement in insulin sensitivity (Pajvani et al, 2004). They report that diabetic patients have a lower HMW/total adiponectin ratio compared with healthy lean individuals and suggest that the new HMW/total ratio index they describe as S_A, would be more useful in cases where differences in insulin sensitivity cannot be explained by differences in absolute adiponectin serum levels alone. Also, of mention is the fact that adiponectin levels increase in normal lean, obese and type 2 diabetic subjects (Yu et al, 2002; Hulstrom et al, 2008; Miyazaki et al, 2004) and this indicates that adiponectin is unlikely to be the only cause of TZD-induced insulin sensitivity (Yu et al, 2002). TZD has emerged as a strong therapeutic tool for diabetes care, but more studies are needed to investigate the dose response effect on adiponectin levels (Yu et al, 2002) and the interactions of glitazones with other PPAR-γ target genes (Hulstrom et al, 2008). The rise in the amount of new cases of diabetes each year makes this an important and essential topic of research, which will help to benefit many and add quality to so many lives affected by this disease.

Figure 1.10: Relative Risks Per 1 Log [mu]g/mL of Adiponectin Level and Type 2 Diabetes Across Studies

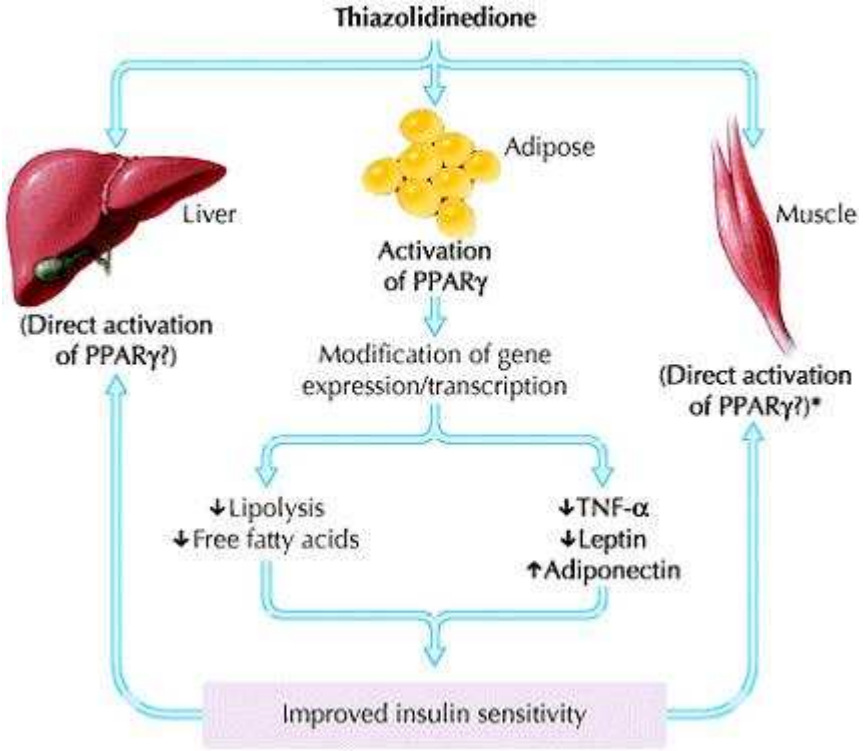
Fourteen data points are included for the 13 studies because results for men and women are shown separately in the Hoorn study (Snijder et al). Size of squares corresponds to the weight of each study in the meta-analysis.



Abbreviations for figure 1.10: CI, Confidence interval

Li et al, 2009 (JAMA)

Figure 1.11: Effects of Thiazolidinedione Medications



Abbreviations for figure 1.11: PPARγ, Peroxisome Proliferator Activated Receptor Gamma; TNFα, Tumour Necrosis Factor Alpha.

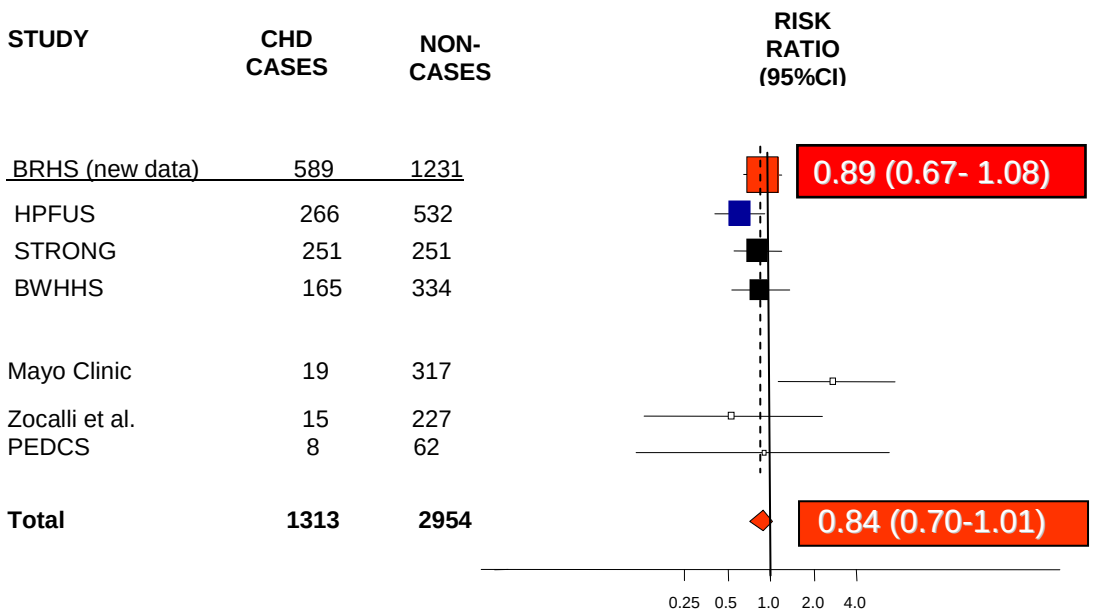
Kumar CKA, 2008 (Recent Pharmaceautical Reviews)

1.2.2 Associations with Cardiovascular Disease Events

There is considerable interest in the vascular actions of adiponectin due to its insulin-sensitising (Sattar et al, 2006), anti-inflammatory (Kadowaki et al, 2005) and anti-atherogenic (von Eynatten et al, 2008) properties. Whilst there is consistent evidence that high levels of adiponectin are associated with lower risk for diabetes, it remains unclear whether high adiponectin is associated with lower risk for CHD events (Sattar et al, 2008). The general assumption was that a reduced adiponectin concentration may underlie the heightened vascular risk associated with obesity and the metabolic syndrome but this association is proving to be more complex that previously thought. In animal models, exogenous adiponectin administration protects against development of atherosclerosis in apolipoprotein E-deficient mice (Yamauchi et al, 2003). In humans, however the evidence has been somewhat conflicting. The Health Professionals Follow Up Study (HPFUS) (Pischon et al, 2004) was the first study to publish results of the relationship between CHD and adiponectin levels and it noted high adiponectin to be independently associated with incidence for myocardial events. They reported that a doubling of baseline adiponectin levels was associated with a statistically significant 20% reduction in MI after adjustments for confounders, which consequently led to the suggestion that adiponectin might be the major mechanistic link between diabetes and increased CHD risk. Since then however, there have been inconsistencies in the data published on this relationship. Some studies (Koenig et al, 2006; Costacou et al, 2005) support the understanding that adiponectin is emerging as an important mediator of future risk for primary CHD while some more recent studies have not been consistent with this analysis (Sattar et al, 2008; Sattar et al, 2006; Lawlor et al, 2005; Lindsay et al, 2005; Kizer et al, 2008). A recent meta-analysis of 7 prospective studies encompassing 1313 CHD cases demonstrated significantly little, if any, association of a single measurement of adiponectin with a combined end point of nonfatal MI and CHD death (Sattar et al, 2008) (Figure 1.12 (Sattar et al, 2008)). So far it has not been elucidated whether adiponectin is a useful prognostic predictor in patients in advanced stages of CHD, thus suggesting that the use of adiponectin for cardiovascular risk is much more complex than previously thought. Lindsay et al suggest that the

relationship of adiponectin to later vascular disease may be less apparent in the presence of Type 2 Diabetes or obesity (Lindsay et al, 2005), and he notes that studies supporting the notion that decreased adiponectin predicts increased risk of CVD, did not include as many participants with diabetes or obesity as his study did. This hypothesis merits further investigation.

Figure 1.12: Prospective Studies of Circulating Concentrations of Adiponectin and CHD Risk



Abbreviations for figure 1.12: BWWHS, British Womens Heart and Health Study; BRHS, British Regional Heart Study; HPFUS, Health Professionals Follow up Study, STRONG, Strength Training Ongoing Study; PEDCS, Pittsburgh Epidemiology of Diabetes Complication Study.

Sattar et al, 2006 (Circulation)

1.2.2.1 HMW Adiponectin and its associations with incident vascular events

Due to the apparent inconsistencies in the data reported from various studies, the separate functioning of the independent parts of the molecule and their biological activity were focused on in more detail. The HMW form of adiponectin is thought to be the key biologically active form of the molecule because it has been shown to be better associated with insulin sensitivity than total adiponectin, has been more strongly linked to a lower risk of incident diabetes, and it has been linked more strongly to protection against endothelial cell apoptosis (Sattar et al, 2008). Commercial ELISA kits for the measurement of the HMW fraction became available and this spurred greater interest in this form of the molecule. A nested case control from the British Women's Heart and Health Study (BWHHS) found no significant link between HMW adiponectin and the risk of incident vascular events, and suggested rather that the molecule was more aligned for the determination of risk with the incidence of diabetes. Von Eynatten et al confirmed this by suggesting from their study that although high total and HMW adiponectin levels were associated with a more favourable CHD risk profile, they were not significantly associated with future secondary CVD events (von Eynatten, 2008).These observations suggest that the use of either total or HMW adiponectin may add no prognostic value to that gained by established cardiovascular risk factors for the occurrence of secondary CVD events (von Eynatten, 2008).

1.2.3 Associations with Coronary Heart Failure (CHF)

Studies in people with prevalent heart failure or acute coronary syndrome (ACS) show that high plasma levels of adiponectin are associated with greater disease severity (McEntegart et al, 2007) (Nakamura et al, 2006) and with higher risk of adverse outcome (Tsutamoto et al, 2007) (Kistorp et al, 2005). B-type natriuretic peptide (BNP) and its inactive N-terminal metabolite NT-proBNP (released in a 1:1 ratio during processing) are sensitive markers of cardiac overload and are markers of prognosis in those with CHF (Clerico et al, 2004). Several studies have shown positive correlations of circulating baseline NT-proBNP and BNP with adiponectin in those with prevalent CHF and

coronary artery disease (Cavusoglu et al, 2006) (George et al, 2006)(Kistorp et al, 2005) (McEntegart et al, 2007) (Nakamura et al, 2006) (Tsutamoto et al, 2007) (von Eynatten et al, 2006). Indeed, in the recent Atherogene Study, baseline adiponectin predicted risk of poor outcome in CHF independent of conventional risk factors, but not independent of BNP (Schnabel et al, 2008). A recent study (Ang et al, 2009) also shows a positive correlation between baseline circulating BNP and adiponectin. In this study increases in plasma adiponectin from baseline, after ACS, to 7 week follow up (rather than absolute levels at each time point), were related to risk of adverse outcome. Although the patterns of risk associations of BNP and adiponectin are different, the data from this study showed that following index admission for ACS, BNP and adiponectin associated with each other at baseline, at 7 week follow up, and that change in both markers also correlated ($r=0.39$, $p<0.001$). The results give insight into the pathophysiology behind increased risk of ACS recurrence, CHF, or death following an index ACS event. The results are suggestive of an interesting and potentially important pathophysiological pathway which links an increase in adiponectin to adverse prognosis in a manner not independent of BNP.

1.2.4 Associations with CVD Mortality and All Cause Mortality

In some studies, high levels of adiponectin have been shown to predict mortality in patients with chronic heart failure (Kistorp et al, 2005; George et al, 2006) and in patients presenting with coronary artery disease (CAD) (Cavusoglu et al, 2006).

The British Regional Heart Study (BRHS), one of the largest studies of its kind, investigated the relationship between adiponectin levels and all cause mortality in elderly men (60-79yrs) with and without CVD (including CHD or stroke). The main aim of this study was to determine whether adiponectin levels were associated with increased CVD mortality in only those with existing vascular disease or heart failure or whether this extended to all individuals in a population. The study findings were consistent with that of previous studies (Kistorp et al, 2005; George et al, 2006); high adiponectin levels are associated with increased mortality in heart failure subjects, but it also extended its findings to show that there is a link between high

adiponectin levels and mortality in the general population of men without diagnosed CVD or heart failure. This increased mortality was seen for cardiovascular and non-cardiovascular causes (Wannamethee et al, 2007). The Rancho-Bernado Study (Laughlin et al, 2007) examined the association of adiponectin with CHD prevalence, incidence, and mortality among 1,513 men and women aged 50-91 years who were followed from 1984-1987 through to 2004. The study also examined the associations of adiponectin with CVD and all cause mortality. Sex differences were evaluated. Adiponectin concentrations were not significantly related to CHD mortality (including after adjustments for age, sex, waist girth, HDL, triglycerides and glucose) and there was no evidence of a trend across adiponectin quintiles. The nature of the adiponectin link to the pathogenesis of CHD and its long term effects is not yet clear.

The study also found that with adiponectin levels in the highest sex-specific quintile, there was a 40% increased risk of CVD death and all cause mortality and this result was independent of age, sex, lipid levels, waist circumference and glucose levels. Therefore although high adiponectin levels have been linked to a more favourable cardiovascular disease risk profile, there seems to be inconsistencies in the fact that high levels seem to be correlated to increased CVD and all cause mortality.

1.2.5 Adiponectin vs. Type 2 Diabetes Mellitus and CVD mortality: Explaining the paradox

A recent editorial (Sattar et al, 2008) explores suggestions as to why an increase in adiponectin levels may be linked to increased CVD mortality. With respect to this, a number of suggestions have been explored which include renal dysfunction and thereby impaired adiponectin clearance, peripheral adiponectin resistance and unintentional weight loss in the elderly. However, Kizer et al have now considered and partially dismissed renal dysfunction as a potential confounder, and although the study relied on self-reported weight loss they also included change in this parameter in their analyses and found the association was independent of weight loss (Kizer et al, 2008). Evidence for the idea that peripheral adiponectin resistance may explain higher levels

in groups at elevated risk, is scarce and this may be an avenue worth exploring.

Sattar and Nelson suggest the possibility that it may be due to reverse causality (Sattar et al, 2008), whereby silent or clinically apparent vascular disease leads to compensatory rises in adiponectin. As mentioned in section 1.1 of this overview, adiponectin synthesis has been proposed to be stimulated in response to vascular inflammation, however systemic markers of inflammation appear to not explain the link between adiponectin and mortality (Kizer et al, 2008). Another suggestion offered by Sattar and Nelson is that a rise in B-Type/Brain Natriuretic Peptide (BNP) levels may link existing vascular disease to higher adiponectin levels because circulating levels of these two parameters show positive correlations in patients with and without chronic heart failure (McEntegart et al, 2007) (Ang et al, 2009). The mechanism is as yet unknown but it has been suggested that high levels of adiponectin in those with acute coronary syndrome (ACS) or heart failure may be a reflection of a salvage mechanism to improve insulin resistance and fatty acid metabolism thereby counteracting vascular stress (McEntegart et al, 2007). Finally we cannot yet rule out the possibility that higher adiponectin levels may actually be harmful to the vasculature and we should bear in mind that measures taken to improve diabetes control or protect against its pathogenicity do not necessarily lessen CVD risk. In order to drive this field forward, future studies are required to better understand the relevance of adiponectin to potential interventions.

1.3 Ethnic Variation

There is evidence of ethnic variation in adiponectin concentrations (Retnakaran et al, 2004). People of South Asian descent (defined as Indian, Pakistani, Sri-Lankan or those having descent from Bangladesh) have an increased risk of Type 2 Diabetes Mellitus compared with any other ethnic groups studied (Retnakaran et al, 2004; Valsamakis et al, 2003; McKeigue et al, 1991). Conventional risk factors associated with the metabolic syndrome (hypertension, dyslipidaemia and smoking) do not fully reconcile the elevated risk this ethnic group faces. Retnakaran et al proposed that there may be additional factors contributing to the increased risk. One candidate molecule

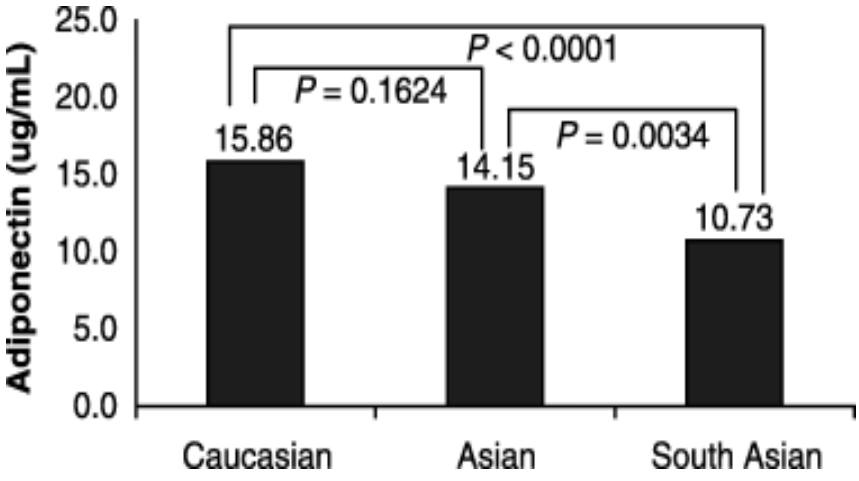
for study is adiponectin, due to its associations with Type 2 Diabetes and CAD. Retnakaran et al reported metabolic characteristics of three main ethnic groups; Caucasian, Asian and South Asian.

180 women who were in their late second or early third trimester of pregnancy were included in the study. Adjusted median adiponectin levels were highest in the Caucasian population, followed closely by the Asians and the lowest levels were recorded in the South Asian population (figure 1.13 (Retnakaran et al, 2004)). Moreover, bearing in mind the inverse relationship between adiponectin and BMI, the observed hypoadiponectinaemia observed in South Asians is particularly striking, since the Caucasian population exhibited a higher mean pregnancy BMI in this study. Similar results were reported by Valsamakis et al in 2003, in a totally different study group. The paper described a reduced adiponectin concentration in 15 middle-aged South Asian males compared with BMI-matched Caucasians. These findings raise the possibility that hypoadiponectinaemia may be a main factor contributing to the increased prevalence of diabetes in South Asians (Retnakaran et al, 2004), and further strengthen the notion of ethically determined differences in adiponectin concentration. Hypoadiponectinaemia is consistent with metabolic features associated within this ethnic group. Central obesity is a common feature in South Asians (McKeigue et al, 1991), and a recent study (Cnop et al, 2003) has shown through body fat distribution analysis, that adiponectin correlates with intra-abdominal fat rather than subcutaneous fat. Also, South Asians exhibit lower levels of HDL-cholesterol than Caucasians (Bhopal et al, 2002) and as mentioned above there is an independent positive association between adiponectin levels and HDL-cholesterol. CRP levels have also been noted to be higher in South Asians (Forouhi et al, 2001) and accordingly since adiponectin is inversely correlated to CRP levels, hypoadiponectinaemia could potentially explain the apparent central role of visceral adipose tissue in the relationship between low-grade inflammation and features of the metabolic syndrome in South Asians (Retnakaran et al, 2004). Retnakaran et al showed that the expected inverse relationship between adiponectin and fasting insulin was observed in Caucasians and Asians, but not in the South Asian population studied. A plausible explanation for this is that ethnically determined hypoadiponectinaemia may obscure the relationship between adiponectin and insulin resistance (Retnakaran et al,

2004). Another study (Ferris et al, 2005), which reported reduced adiponectin levels in black South Africans and Asian Indians compared with BMI-matched Caucasians, confirmed these same findings (table 1.2). Within each ethnic group, adiponectin only correlated inversely with HOMA in Caucasian subjects, however when all ethnic groups were combined, multiple regression analysis demonstrated that serum adiponectin levels corrected for BMI and ethnicity did not correlate with HOMA. Therefore the indication from this particular study is that ethnic variation in insulin sensitivity is not dependent on serum levels of adiponectin and that the relationship between adiponectin and insulin resistance varies across ethnic groups (Ferris et al, 2005).

Figure 1.13: Adjusted Mean Adiponectin in Pregnant Women by Ethnic Group.

Adjusted for age, fasting insulin, glucose intolerance, pre-pregnancy body mass index, weight gain during pregnancy, previous history of gestational diabetes mellitus, and family history of diabetes mellitus



Retnakaran et al, 2004 (Diabetic Medicine)

Table 1.2 Demographic and biochemical data for Ferris's Study

Data is expressed as means ± SD; *p < 0.01 versus Asian subjects, † p < 0.01 versus white subjects.

Variables	Asian	Black	White
Age (years)	41.5 ± 10.8	36.2 ± 11.3	37.9 ± 9.0
BMI (kg/m2)	27.3 ±5.0	27.6 ± 5.3	26.2 ± 4.6
Waist-to-hip ratio	0.86 ±0.09	0.78 ± 0.07*	0.82 ± 0.10*
Triglyceride (mM)	1.77 ±1.15	0.92 ± 0.75*†	1.34 ± 0.88
HDL (mM)	1.29 ± 0.29	1.36 ± 0.32	1.41 ± 0.33
LDL (mM)	3.28 ± 1.06	2.39 ± 0.65*†	3.29 ± 1.05
Cholesterol (mM)	5.33 ± 1.24	4.10 ± 0.76*†	5.30 ± 1.01
Adiponectin (µg/ml)	5.86 ± 2.50†	5.71 ± 2.50†	8.11 ± 4.39
HOMA	3.41 ± 2.85†	2.62 ± 0.99†	1.76 ± 0.63

Abbreviations for table1.2: BMI, Body Mass Index; HDL, High Density Lipoproten; LDL, Low Density Lipoprotein; HOMA, Homeostasis Model Assessment

Ferris et al, 2005 (Horm. Metab. Res.)

1.4 Aims and Objectives of Studies Reported in This Thesis

The aim of the work documented in this thesis was to review literature on, and explore the complex functioning of the adiponectin molecule in human health and disease. There were two specific aims: i) I considered the methodologies behind the collection, processing, measurement and analysis of total and HMW adiponectin to assure that future research into this molecule within our laboratory and elsewhere was robust, and ii) I examined adiponectin's relationship to CVD both in terms of prediction and subsequent outcome following an event.

The role of adiponectin in human health and disease is more complex than originally thought and it may be that the molecule exhibits separate functioning in times of stress within the body and with normal human health. The thesis aims to add insight to this hypothesis.

Chapters three and four focus on Methodological considerations. Many factors other than disease influence the results of tests within a laboratory. These factors may be pre-analytical, analytical or post-analytical (or a combination of these). Whenever possible, these variables should be controlled in order to minimize their effect on test outcome. Pre-analytical variables are variables associated with the patient, sample collection and sample handling. These generally affect the composition of the body fluid **before** analysis. Analytical variables are factors which influence the analytical procedure and post-analytical variables involve the different ways data from the laboratory is presented, stored and transferred to the clinician. This thesis focuses on pre- analytical and analytical variables only.

Chapters five and six report findings from two studies published on work carried out within our laboratory or in collaboration with other local research facilities, where I had a role in assessment and drafting of relevant articles. These chapters focus on the role adiponectin plays in disease. They aim to add insight into the complex functioning of the adiponectin molecule with specific focus in chapter 6 on ways it may express its role through interactions

with other molecules that correlate to its levels at different stages in the pathogenesis of heart disease.

Aims of Chapter 3.2 – Effect of Freeze/Thaw Cycles

The aim of this study was to investigate the effect of repeated freeze/thaw on the amount of measurable concentrations of adiponectin in a blood sample. This study is important to ensure that samples are not compromised with continual freeze/thaw.

1.4.2 Aims of Chapter 3.3 – Blood Processing

The aim of this study was to determine if leaving bloods un-separated (a) in a fridge/cold room at 7˚C and (b) on a bench at room temperature (23˚C - 25˚C) for up to 6 days after venipuncture would have an effect on measurable concentrations of total adiponectin in EDTA blood samples. This is an important variable to assess because, at times, it is not possible to get bloods from the venue of venipuncture to the laboratory for analysis right away.

1.4.3 Aims of Chapter 3.4 – Biological Variability

The aim of this study was to investigate biological variation of total adiponectin and therefore assess whether total adiponectin levels vary significantly in an individual (human) over the period of 6 weeks.
This information will help where a study may dictate a patient/volunteer to give a sample for weekly analysis. It will help to eliminate any variables associated with natural biological change in adiponectin concentrations over time.

1.4.4 Aims of Chapter 4.2 – Measurement of Citrate Samples on a Total Adiponectin ELISA

The aim of this study was to validate samples that have been preserved with citrate anticoagulant for use in a total adiponectin R&D systems ELISA assay (general methods chapter 2.4.1.1).

1.4.5 Aims of Chapter 4.3 – Comparison of Commercial Adiponectin ELISA Assays

The aim of the study was to measure total adiponectin using the ALPCO diagnostics ELISA method, the Mercodia adiponectin ELISA method and the R&D Systems ELISA method on the same set of samples. I wanted to determine whether the results obtained were comparable between methods.

1.4.6 Aims of Chapter 5 - High Molecular Weight Adiponectin and Incident Coronary Heart Disease: Findings from a Prospective Case Control Study Nested within the British Women's Heart and Health Study (BWHHS)

This study was designed to test whether HMW adiponectin (rather than the previously reported total adiponectin from the original BWHHS (Lawlor et al, 2005)) was better associated with risk for incident vascular events in older women. There has been some speculation that the HMW fraction of adiponectin was the more biologically active part of the molecule. This study adds insight to this hypothesis.

1.4.7 Aims of Chapter 6 - Serial Changes in Adiponectin and BNP in ACS Patients: Paradoxical Associations with Each Other and with Prognosis.

The aim of this study was to further investigate the potential pathological link between BNP and adiponectin. By examining the inter-relationship of circulating adiponectin and BNP both at baseline and at a 7 week follow up, the aim was to determine whether change in either parameter was a better predictor of adverse events than a single measurement. Also, changes in concentrations of adiponectin over time were investigated to see if they correlated with changes in BNP over time.

Chapter II: General Methods

2.1 Review of the Literature

PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) was used for the purpose of literature searches in this thesis. It is a service of the U.S. National Library of Medicine that includes over 19 million citations from MEDLINE and other life science journals for biomedical articles back to 1948. PubMed includes links to full text articles and other related resources (Pubmed online).

Key word searches were used including: Adiponectin, Adiponectin in: Cardiovascular disease, Diabetes, Insulin sensitivity, Health and disease, Heart Failure, Ischemic Heart disease, Males, Females, Children.

Results were restricted to English language, and for epidemiological studies data were restricted to human subjects as well.

The University of Glasgow library and the departmental book shelf (including books on related topics and past theses) were used to research books on this topic.

2.2 Subject Recruitment and Sample storage

2.2.1 Subject Recruitment

Each study in this thesis comprises a different sample set and for this reason the recruitment of healthy volunteers and patients, venipuncture procedures and sample handling and processing are described in detail in each relevant chapter.

2.2.2 Sample Storage

Accuracy of results is a reflection on the care taken in sample storage.

All samples used in the studies presented in this thesis are of a high quality and the history of the samples is well documented. All samples handled in our

own laboratory (Chapters 3+4) are frozen immediately after processing at -80˚C and removed and snap thawed (37˚C) immediately before assay. Samples handled in multi-centre laboratories (Chapters 5+6) are done so in a standardised manner by professionally trained researchers according to good laboratory practice (GLP) (http://www.mhra.gov.uk/Howweregulate/Medicines/Inspectionandstandards/GoodLaboratoryPractice/index.htm).

2.3 Materials

Names and addresses of manufacturers and suppliers of kits and reagents, hardware and software used for the studies in this thesis are given in the appendix 1. All kit inserts are available in appendix 3.

2.4 Laboratory Methods

2.4.1 Measurement of Total Adiponectin

2.4.1.1 R&D Systems ELISA Method

Total Adiponectin concentrations were measured using the R & D Systems Quantikine Human Adiponectin/Acrp30 Immunoassay (Catalog Nos DRP300). See the R&D systems kit insert in **appendix 3** for assay instructions. Specific equipment and quality controls used by our laboratory during the analysis on this kit are listed below, as well as methods for the calculation of absorbencies.

SAMPLE

EDTA Plasma was used in this thesis for analysis involving this kit, except where specified, in chapter 4.2 for the validation of citrate plasma and in chapter 4.3, where serum was used for analysis.

EQUIPMENT

- Thermo Lab systems Multiskan Ascent Microplate reader capable of measuring absorbance at 450nm with correction wavelength set at 540nm or 570nm
- Ascent software package
- Gilson pipettes and pipette tips
- Thermo-systems Finnpipette (50μl-300μl) multi-channel pipette
- 500ml graduated cylinder
- Squeeze bottle
- Electronic timer clock
- Plastic plate sealers supplied with the kit

QUALITY CONTROL

An internal laboratory quality control (EDTA plasma) was used to make sure the assay was running effectively.

CALCULATION

All calculations were done using the Multiskan Ascent Software Package. Analysis was repeated when results fell out-with the assay range.

2.4.1.2 Mercodia Adiponectin ELISA Method

Total adiponectin concentrations in chapter 4.3 were also measured using the Mercodia Adiponectin ELISA assay 10-1193-01. See the Mercodia kit insert in **appendix 3** for assay instructions. Specific equipment and quality controls used by our laboratory during the analysis on this kit are listed below, as well as methods for the calculation of absorbencies.

SAMPLE

Serum samples were used for all analysis in this thesis with this kit.

EQUIPMENT

- Fisherbrand Whirlimixer
- Thermo Denley minimix plate shaker

- Thermo Lab systems Multiskan Ascent microplate reader capable of measuring absorbance at 450nm
- Ascent software package
- Gilson pipettes
- Thermo-systems Finnpipette (50µl-300µl) multi-channel pipette
- 1000mL graduated cylinder
- Squeeze bottle
- Electronic timer clock
- Gilson repeater pipette and tips
- Plastic plate sealers supplied with the kit

QUALITY CONTROL

Mercodia Obesity Control A, B, C/Human is designed to be used as a three-level control for Mercodia adiponectin ELISA. It is manufactured from Human serum. Each Mercodia Obesity Control kit contains 3 vials of lyophilised serum, each containing 0.5 mL after reconstitution.

Ranges: A = 5.5 – 8.5 ug/mL
B = 6 – 11 ug/mL
C = 11 – 19 ug/mL

Internal quality controls with low, intermediate and high adiponectin concentrations should be routinely assayed as unknowns, and results charted from day to day.

An internal laboratory quality control (EDTA plasma) was also used on each assay.

CALCULATION

All calculations were done using the Multiskan Ascent Software Package. Analysis was repeated when results fell out-with the assay range.

2.4.2 Measurement of High Molecular Weight Adiponectin

High Molecular Weight adiponectin (and total adiponectin – studies 3.2 and 4.3) concentrations were measured using the American Laboratory Products Company (ALPCO) Diagnostics: Adiponectin (Multimeric) EIA (Catalog Number 47-ADPH-9755). See the ALPCO Diagnostics kit insert in **appendix 3** for assay instructions. Specific equipment and quality controls used by our laboratory

during the analysis on this kit are listed below, as well as methods for the calculation of absorbencies.

SAMPLE

Serum samples were used for analysis in chapters 3.2 and 4.3.
EDTA Plasma was used in chapter 5.

EQUIPMENT

- Heating block (temp = 37˚C)
- Fisherbrand whirlimixer
- Versamax microplate reader capable of measuring absorbance at 492nm with correction wavelength set at 600-700nm
- Softmax Pro computer software package for calculations
- roller mixer (to ensure thorough mixing of pre-treatment buffer and protease solutions I and II)
- Gilson pipettes and pipette tips
- Thermo-systems Finnpipette (50µl-300µl) multi-channel pipette
- 1000mL graduated cylinder
- Squeeze bottle
- Electronic timer clock
- Gilson Repeater pipette and tips
- Plastic plate sealers supplied with the kit

QUALITY CONTROL

An internal laboratory quality control (EDTA plasma) was used to make sure the assay was running effectively.

CALCULATION

All calculations were worked out by creating a standard curve using Softmax Pro 3.12 computer software.

2.5 Quality Control

A pooled EDTA plasma sample was used to monitor inter-assay quality control (QC). Blood from 2 healthy volunteers from the Vascular Biochemistry

laboratory was drawn into 9mL EDTA vacutainers (BD Vacutainer Systems: Becton, Dickinson and Company). Blood was separated immediately after venipuncture by spinning (3000rpm at 4˚C for 10 mins) in the under-bench centrifuge (Beckman Coulter, GS-6KR) and aliquoting 500µl plasma into apex tubes. These tubes were clearly labelled as controls and immediately frozen at -80˚C until required.

For ELISA assays, 2 wells at the end of each assay plate were filled with fresh QC. The mean of the values from these two aliquots was determined and the process repeated over 22 separate runs on R&D systems total adiponectin ELISA assays (Chapter 2.4.1.1) and used to calculate out an inter-assay coefficient of variation (CV). The CV was calculated out to be 6.73 %. This is in line with the published inter-assay precision given in the R&D adiponectin ELISA standard operating procedure (SOP) (appendix 3) where the CV (%) < 7.0. This Quality control was also used to calculate out an intra-assay CV on each run, and this was in each case < 4.5% which is again in line with the recommendations given by R&D systems for the ELISA method, where the intra-assay CV is < 4.7%.

2.6 Statistical Analysis

For all pre-analytical and analytical variables I calculated basic statistics (mean, median, CV, standard deviation (SD)) using an Excel spreadsheet.

For all other more complex statistics (excluding the intra-class correlation coefficients (chapter 3.4)) the website for IFA statistics was used (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on those in the paper by Ernst et al, 1985.

To maintain equivalence throughout the thesis, we assumed non-normal distribution for **all pre-analytical and analytical variable studies (Chapters 3+4)** and used non-parametric tests to carry out statistical evaluation on our data in these studies. The pre-analytical and analytical studies (chapters 3+4) presented in this thesis are not large enough in sample number to assess the true skewedness of the distribution. It is acknowledged that a non-parametric

test with data from a small sample population with a wide range of values may overestimate the significance level. The non-parametric tests may lack statistical power with small sample numbers. However, from the majority of literature reviewed on adiponectin distribution, the results are mostly highly skewed (Sattar et al, 2006; Cavisoglu, 2006) and therefore we have assumed non-normal distribution. The Wilcoxon matched pairs signed ranks test was used in chapters 3.2 - 3.4 and 4.2 - 4.3 to determine significance, and the Spearman's rank correlation coefficient was also used in these studies to determine whether there was a preservation in the given order between the two variables.

The **Wilcoxon matched pairs signed-rank test** is a non-parametric statistical hypothesis test for the case of two related samples or repeated measurements on a single sample. It can be used as an alternative to the paired Student's t-test when the population cannot be assumed to be normally distributed. It should be used whenever the distributional assumptions that underlie the *t*-test cannot be satisfied (median = mean).

The **Spearman's rank correlation coefficient (ρ)** is a non-parametric measure of correlation (wikipedia). It assesses if there is a monotonic relation between two variables.

The **intra-class correlation coefficients** presented in the Biological Variability chapter 3.4, were calculated out on an Excel spreadsheet according to Fleiss (1986). This test is used to determine the optimum number of measurements required for an assay to reduce misclassification of individuals (Poorhang, MSc, 2005). Dr. Paul Welsh offered his expertise and guidance in the calculation of these values.

In the clinical studies chapters 5+6, where sample numbers were much larger, distribution was tested for and where appropriate, values were log-transformed to follow normal distribution.

Chapter III: Methodological Considerations - Effects of Pre-Analytical Variables on Measurements of Adiponectin

3.1 Introduction to pre-analytical variables

Many variables can affect the results obtained from analysis of blood samples in both the clinic and laboratory environment. Pre-analytical variables are those variables that may affect sample viability before it is analysed. These include season, diet, time of day, stress, and physical activity of the patient, as well as encompassing technical aspects of blood collection such as venipuncture technique, anticoagulation, processing, and storage of blood samples (Welsh, PhD, 2008).

The ELISA method for determination of adiponectin has become an increasingly popular and accurate way of measuring levels of adiponectin in human blood. It is cost effective and easy to perform, with samples being analysed and results being obtained on the same day. However, before one can use the results obtained from this method in proposing new thoughts on the molecule's functioning in the body, we have to be sure the results obtained are not tainted with potential confounders before analysis. Therefore it is essential to understand the steps that must be taken in order to maintain a high standard of sample quality.

Due to time constraints it was not possible to investigate all potential confounding pre-analytical factors, however from the series of studies presented in this chapter the aim is to show that adiponectin results presented in the clinical/epidemiological study chapters of this thesis can be validated accordingly.

This chapter investigates

- Freeze/thaw cycles and the effects this stress may have on measurable concentrations of adiponectin in a sample.
- The effect of time taken between venipuncture and separation and the temperature a sample may be subjected to during processing and before freezing.
- Biological variability in study samples within our laboratory.

3.2 Effect of Repeated Freeze/Thaw on Adiponectin Levels in Human Blood Samples

3.2.1 Introduction

Large study cohorts often require several analytes to be measured on their limited sample volumes (often only 1 aliquot is available for analysis), and so it is not always possible to do all the tests on the first sample defrost. Some assays can take 1-2 days to complete and for this reason it may be necessary to repeatedly thaw and refreeze the samples on several occasions to allow all measurements to be completed. Some peer reviewed studies suggest both total adiponectin and HMW adiponectin to be fairly robust, and showed that up to five freeze/thaw cycles had no effect on the measurable concentrations in plasma (Schraw et al, 2008; Tanita et al, 2008). The robustness of this molecule may reflect the reason behind the use of ELISA assays in many epidemiological studies; ELISA antibody detection only requires the antigenic site to be intact, rather than the marker show biological activity (Welsh, PhD, 2008). In this study we address the issue of whether this repeated freeze/thaw compromises the measureable concentrations of adiponectin in the plasma.

3.2.2 Principle

The aim was to investigate the effect of repeated freeze/thaw on the amount of measurable adiponectin in a sample. We also aim to investigate if measurable concentrations of adiponectin maintain rank correlation with freeze thaw cycles.

A pilot study was first of all set up examining the effect of 7 freeze/thaw cycles on total and HMW adiponectin levels in 5 **serum** samples from the Reykjavik[3] cohort. After this data was analysed, a larger study group of 13

[3] Aims to examine risk factors for out-of-hospital cardiac arrest. It is a long-term, prospective, population-based cohort study that started in 1967. Methods and results from 1987 to 1996, 137 men and 44 women out of the 8006 men and 9435 women in the study sustained out-of-hospital cardiac arrest due to cardiac causes.

people was used to study the effects of 5 freeze/thaw cycles on total adiponectin levels in **plasma** samples.

3.2.3 Pilot Study (Reykjavik cohort)

3.2.3.1 Pilot Study Subjects and Methods

Blood collection, storage and analysis

The Reykjavik Study, initiated in 1967 as a prospective study of cardiovascular disease, has been described elsewhere previously (Danesh et al, 2004). A total of 8888 men born between 1907 and 1934 and 9681 women born between 1908 and 1935 who were residents of Reykjavik, Iceland, and its adjacent communities on December 1, 1966 were enrolled in the study. All participants had no previous history of myocardial infarction. 9 nurses administered questionnaires, made physical measurements, performed spirometry and electrocardiography, and collected venous blood samples after an overnight fast for the measurement of the erythrocyte sedimentation rate and to prepare aliquots of serum, which were stored at -20°C for subsequent analysis. A set of 5 samples randomly selected from this cohort were used to test the effect of freeze/thaw on the measurable concentrations of adiponectin in serum samples. The Reykjavik samples had previously been defrosted twice for use in the original Reykjavik study. Five samples from the cohort were randomly selected and 250µl from each was aliquoted into a separate apex tube. These apex tubes were labelled with sample ID, date and control, and subsequently frozen at -80°C.
Over the next four consecutive days the original serum sample from the cohort was defrosted at room temperature and refrozen. After the final defrost on the 5th day an aliquot from each serum sample was removed into an apex tube, labelled with freeze/thaw aliquot and stored at -80°C for analysis. At the end of the pilot study defrosts, and including the previous defrosts from the original Reykjavik Study, these samples had undergone a total of seven defrosts.

At the time of analysis, the apex tubes were removed from the freezer and snap thawed at 37°C. HMW adiponectin and total adiponectin concentrations

were measured on all serum samples using the ALPCO Diagnostics Adiponectin ELISA kit (General Methods 2.4.2). The original control samples along with the freeze/thaw aliquots from this study were tested on the same plate, thus avoiding any inter-assay variation. All available data was analysed.

Statistical analysis

Means, Medians, 25^{th} percentiles and 75^{th} percentiles (Inter quartile ranges (IQR)) were calculated using an Excel spreadsheet. Dr. Paul Welsh advised on statistical analysis. The Wilcoxon Matched-Pairs Signed-Ranks Test was used to establish p-values and the Spearman's rank Correlation Test was used to establish ρ-values. (Chapter 2, General Methods). The website for IFA statistics was used for these tests (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on the book by Fleiss in 1986.

3.2.3.2 Results

Results for this pilot study are summarized in tables 3.1 –3.3

Tables 3.1 (Total adiponectin) and **3.2** (HMW adiponectin) summarise the effects of freeze/thaw on the 5 pilot study samples from the Reykjavik cohort. Medians, 25^{th} percentiles and 75^{th} percentiles are tabulated for both the baseline samples/control samples (2 defrosts) as well as for the samples after they were subjected to an additional 5 defrosts.

Table 3.3 shows the p-values and correlation coefficients obtained from the data. Because the sample size was so small it was hard to assess whether the data was skewed, so a non-normal distribution was assumed and the Wilcoxon Matched-Pairs Signed-Ranks Test was used. P-values obtained were non-significant. The Spearman's rank correlation was used to calculate ρ-values and these show a good correlation between the baseline and the freeze/thawed samples. The HMW adiponectin value was borderline significant for this test ($p<=0.08$).

Figure 3.1 (total adiponectin) and **3.2** (HMW adiponectin) show measured levels at baseline and after the freeze/thaw cycles.

Table 3.1: Summary of the Data for Total Adiponectin Concentrations at Baseline and After the Freeze/thaw Cycles on the 5 Pilot Study Reykjavik Samples.

Time point	**Median and IQR** (µg/mL) of 5 samples
Baseline	8.11 **IQR** (8.0 – 11.02)
After 7 freeze/thaw cycles	7.78 **IQR** (7.03 – 8.26)

Abbreviations for Table 3.1: IQR, Inter-quartile range.

Table 3.2:Summary of the Data for HMW Adiponectin Concentrations at Baseline and After the Freeze/thaw Cycles on the 5 pilot Study Reykjavik Samples.

Time point	**Median and IQR** (µg/mL) of 5 samples
Baseline	5.53 **IQR** (3.62 - 6.01)
After 7 freeze/thaw cycles	5.94 **IQR** (4.70 - 6.15)

Abbreviations for Table 3.2: IQR, Inter-quartile range.

Table 3.3: P-values and Spearman's Rank Correlation Coefficients for Baseline vs. Freeze/thaw Sample for Both Total and HMW Adiponectin Concentrations

	Total adiponectin	HMW adiponectin
Wilcoxon matched-pairs signed-ranks test	$p = 0.06$	$p = 0.44$
Spearman's rank correlation coefficient	$\rho = 1$, $p = 0.02$	$\rho = 0.9$, $p = 0.08$

Figure 3.1: Graph Showing Concentrations of Total Adiponectin Measured in the Reykjavik Samples at Baseline and After Seven Freeze Thaw Cycles

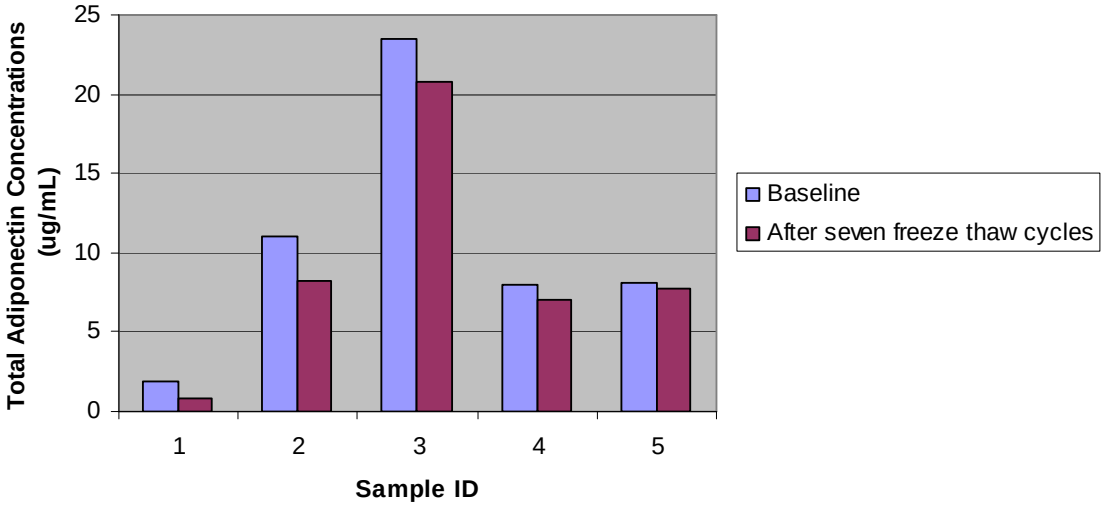
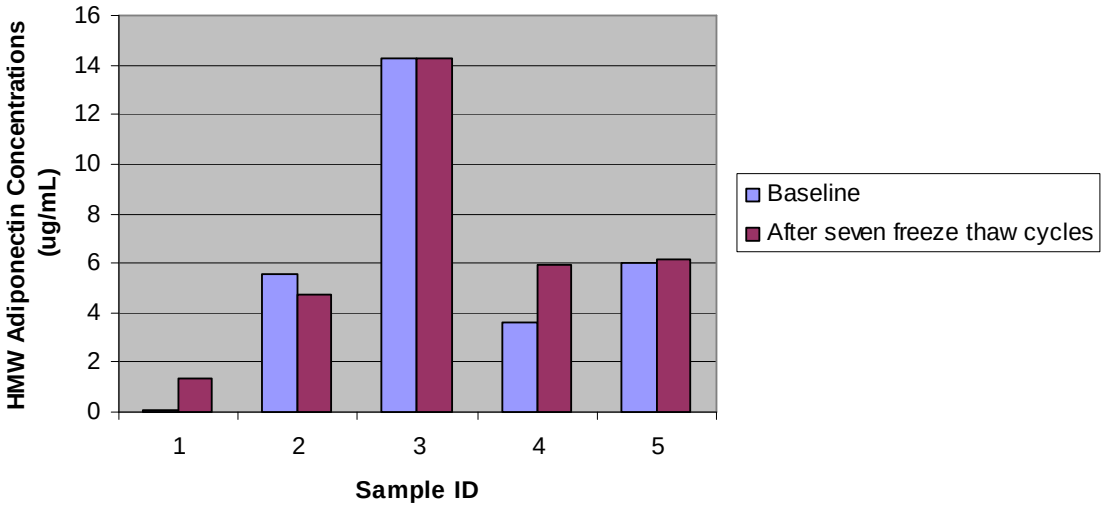


Figure 3.2: Graph Showing Concentrations of HMW Adiponectin Measured in the Reykjavik Samples at Baseline and After Seven Freeze Thaw Cycles



3.2.3.3 Discussion of pilot study

There was no significant change in concentrations of adiponectin after 7 freeze thaw cycles ($p = 0.06$, total adiponectin; $p = 0.44$, HMW adiponectin) and these samples appear to show very good correlations with the control/baseline samples ($\rho = 1$, total adiponectin; $\rho = 0.9$, HMW adiponectin). Our results suggest that both total and HMW adiponectin are therefore stable for up to seven freeze thaw cycles and these findings corroborate with a recent study (Tanita et al, 2008) which found that up to five freeze/thaw cycles had no significant influence on the measurable concentrations of HMW adiponectin in serum samples.

However these results must be taken in earnest as we must still bear in mind that the sample set was small and that larger studies are needed to corroborate our result. Small data sets can present a dilemma; it is difficult to tell if the data come from a normally distributed population. The non-parametric tests are less powerful and the parametric tests are less robust within smaller data sets. This fact may attribute to the borderline significant p value that was obtained for the HMW adiponectin samples. It is not common practice to attribute great belief in a result which has been drawn from a small sample set and so I acknowledge that a larger sample set may provide more insight into this variable and should be pursued.

Limitations of the study

One of the major limitations with a small study sample set is the fact that one cannot determine whether the data is normally distributed and therefore statistical analysis has less impact in terms of parametric associations. In recent published literature (Sattar et al, 2008; McEntegart, 2007) adiponectin results are generally skewed and so for the statistical analysis in this study we have assumed a non-normal distribution, and used non-parametric tests on the raw data. Also, in this study, analysis was only carried out on the baseline sample and the sample after it had undergone 7 freeze/thaw cycles. Concentrations were not measured after each thaw. This was because it was a

pilot study and was set up merely to assess if there was a potential for samples to deteriorate under this stress.

By defrosting a larger sample set (n = 13) each day for five consecutive days and storing an aliquot from each defrost, we can determine better if there is any compromise in the concentrations of adiponectin in the sample, and if so, at which point the sample starts to deteriorate.

3.2.4 Effects of freeze/thaw on measurable concentrations of total adiponectin in a larger study group (n=13)

3.2.4.1 Study subjects and methods

Blood collection, storage and analysis

13 healthy volunteers, 9 female and 4 male (age 26 – 58 yrs), for this study were obtained from the Vascular Biochemistry laboratory at the Glasgow Royal Infirmary. Volunteers in the study signed an informed consent form (Appendix) giving permission for a trained phlebotomist to take blood. They were also informed that their bloods would be used for research purposes and stored on the 4th Floor of the Queen Elizabeth building at the Glasgow Royal Infirmary. At the time of bleeding, 1 x 9mL EDTA vacutainer (BD Vacutainer Systems: Becton, Dickinson and Company) was taken from these volunteers by a trained phlebotomist. The volunteers were not fasted. After venipuncture the blood samples were centrifuged (3000rpm, at 4˚C, for 10 mins) in the under bench centrifuge (Beckman Coulter, GS-6KR) within 30 mins. The separated plasma was aliquoted into blue eppendorf tubes (2 x 1.5mL). These tubes were clearly labelled with sample ID, date, time of venipuncture and name of study. One of the eppendorfs per volunteer was used as the baseline/control sample and stored at -80˚C, while the other was removed and snap-thawed each day at 37˚C over 5 consecutive days. On each day an aliquot was taken from the sample and stored as 1st thaw, 2nd thaw, 3rd thaw, 4th thaw and 5th thaw consecutively. The samples were then kept stored at -80˚C until analysis could be carried out. On the day of analysis the samples were snap thawed at 37˚C. The samples were tested for total adiponectin using the R&D Systems ELISA method (General Methods Section 2.4.1.1). All samples were measured on the same assay plate in order to avoid inter-assay variation. Due to financial constraints HMW adiponectin was not tested for on this sample set. All available data was analysed.

Statistical analysis

Medians, 25th percentiles and 75th percentiles were calculated using an Excel spreadsheet. Dr. Paul Welsh advised on statistical analysis. The Wilcoxon Matched-Pairs Signed-Ranks Test was used to establish p-values and the Spearman's rank Correlation Test was used to establish ρ -values (Chapter 2, General Methods). The website for IFA statistics was used for this test (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on the book by Fleiss in 1986.

3.2.4.2 Results

Results for this study are summarized in **tables 3.4 and 3.5.**

Table 3.4 shows Medians, 25% IQR's, and 75% IQR's for total adiponectin concentrations at the 6 time points for all 13 volunteers. As can be seen from the table values, the median remains roughly the same throughout the 5 freeze/thaw cycles.

Table 3.5 summarises p- values and ρ – values over the 5 freeze/thaw cycles. Rank correlation is maintained throughout the 5 cycles and there was no significant change between baseline values and those obtained at thaw 1, 3, 4 and 5. Baseline values compared to thaw 2 values show a significant change, however rank order is still maintained on this thaw.

Figure 3.3 graphically shows the total adiponectin median levels over the five freeze/thaw cycles.

Table 3.4: Summary of Data for Total Adiponectin Over the 5 Freeze/thaw Cycles.

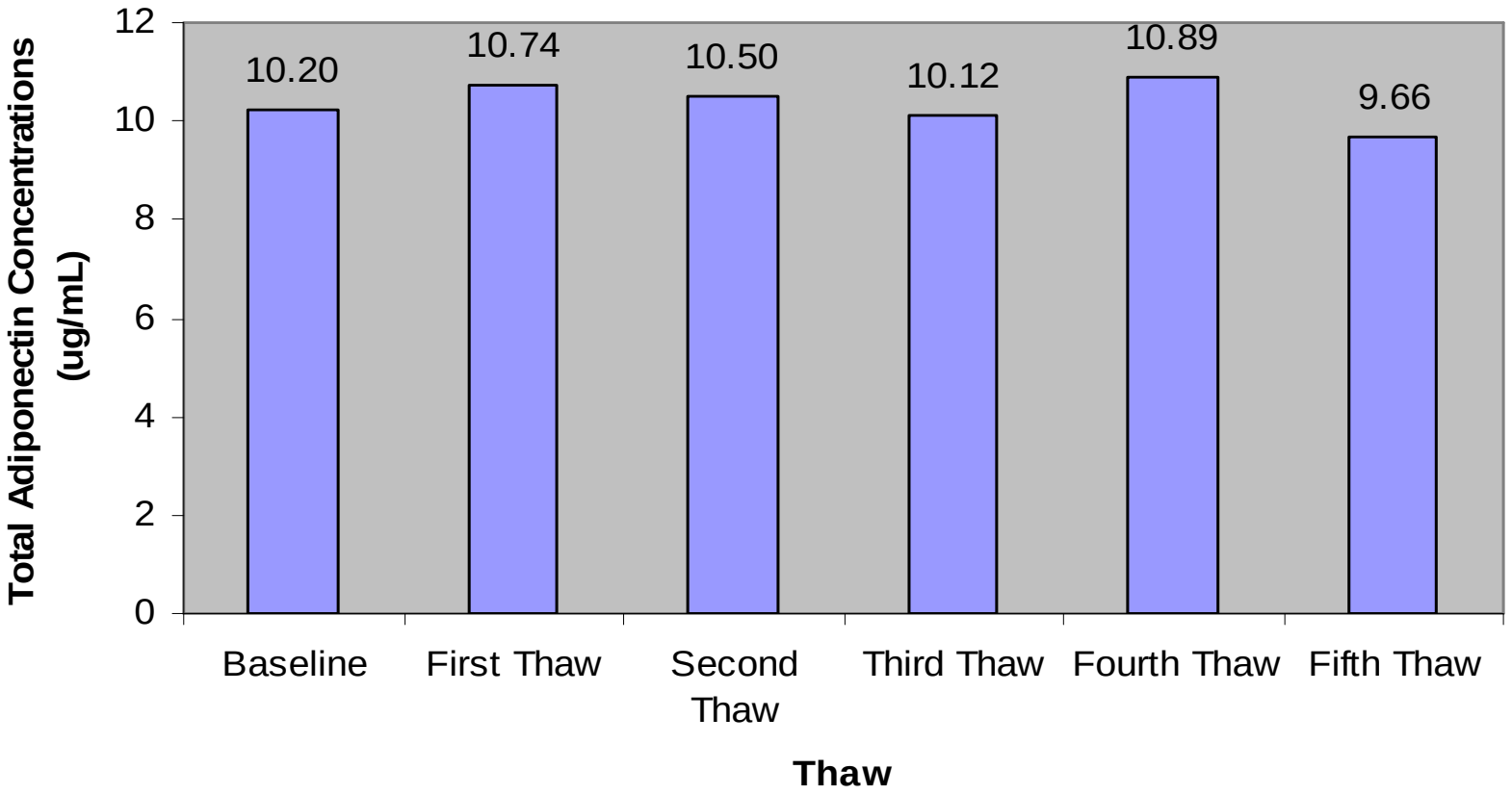
	Baseline	1st Thaw	2nd Thaw	3rd Thaw	4th Thaw	5th Thaw
Adiponectin Median and IQR for 13 samples (µg/mL)	10.21 **(IQR)** 6.57 – 11.84	10.74 **(IQR)** 6.73 – 14.40	10.50 **(IQR)** 7.72 – 14.04	10.12 **(IQR)** 7.30 – 13.05	10.89 **(IQR)** 7.38 – 11.89	9.66 **(IQR)** 7.41 – 13.39

Abbreviations for Table 3.2.4: IQR, Inter-quartile range.

Table 3.5: P-values and Spearman's Rank Correlations for Total Adiponectin Measurements Over the 5 Freeze/thaw Cycles.

	Baseline vs. 1st Thaw	Baseline vs. 2nd Thaw	Baseline vs. 3rd Thaw	Baseline vs. 4th Thaw	Baseline vs. 5th Thaw
Wilcoxon matched-pairs signed-ranks test	$p = 0.50$	$p = 0.01$	$p = 0.95$	$p = 0.45$	$p = 0.96$
Spearman's rank correlation coefficient	$\rho = 0.89$	$\rho = 0.98$	$\rho = 0.98$	$\rho = 0.95$	$\rho = 0.99$

Figure 3.3 Total Adiponectin Median Levels Over the Five Freeze/Thaw Cycles



3.2.4.3 Discussion

The manufacturers (R&D systems, ALPCO Diagnostics) of the assay kits used in this study recommend avoidance of repeated freeze/thaw cycles but if plasma samples need to frozen before analysis and thawed again, they should be done so speedily after venipuncture and snap-thawed at 37˚C to avoid denaturation of protein in the sample. Manufacturers do not always specify the number of thaws the sample will be stable to on the particular assay.

This study shows that up to five freeze thaw cycles has no significant effect on the concentrations of total adiponectin in human plasma. Figure 3.3 illustrates that median levels of total adiponectin do not change significantly over the five freeze thaw cycles.

It must be highlighted that the comparison between the baseline samples and the samples at the 2^{nd} thaw showed a significant change ($p = 0.01$) (table 3.5), however the correlation remained strong. Therefore it can be concluded that although there may be a statistically significant change in the values at thaw 2, all samples in the set still maintained rank order and therefore correlate accordingly to the values at baseline.

I conclude from the results presented here that total adiponectin is suitable to be used in large epidemiological studies where several freeze thaw cycles are required before analysis. More data is required using a larger population to confirm and further expand on these findings.

3.3 Blood Sample Processing

3.3.1 Introduction

Both in routine and research laboratories, it is not always possible to analyse blood samples right away, and sometimes samples can sit on a bench or in a fridge for some time before being centrifuged, separated and analysed or frozen for storage, especially during busy times. Also, blood samples can be sent by post or transferred from doctors or nurses practices' and these are not always delivered the same day. Under good laboratory practice, blood samples should be centrifuged for separation within 30 minutes of venipuncture, however this is not always possible i.e. when you are not taking blood in the vicinity of a laboratory. It is common practice for some tests to keep blood bottles refrigerated or on ice if they cannot be centrifuged and separated right away. This minimizes breakdown of specific molecules in the blood. This section of chapter 3 investigates whether measurable concentrations of adiponectin deteriorate in blood samples that have been left as whole blood on a bench between 23˚C - 25˚C or in a fridge between 4 - 8˚C for up to 6 days. This study allowed assessment of the robustness of adiponectin to differing modes of sample collection.

3.3.2 Principle

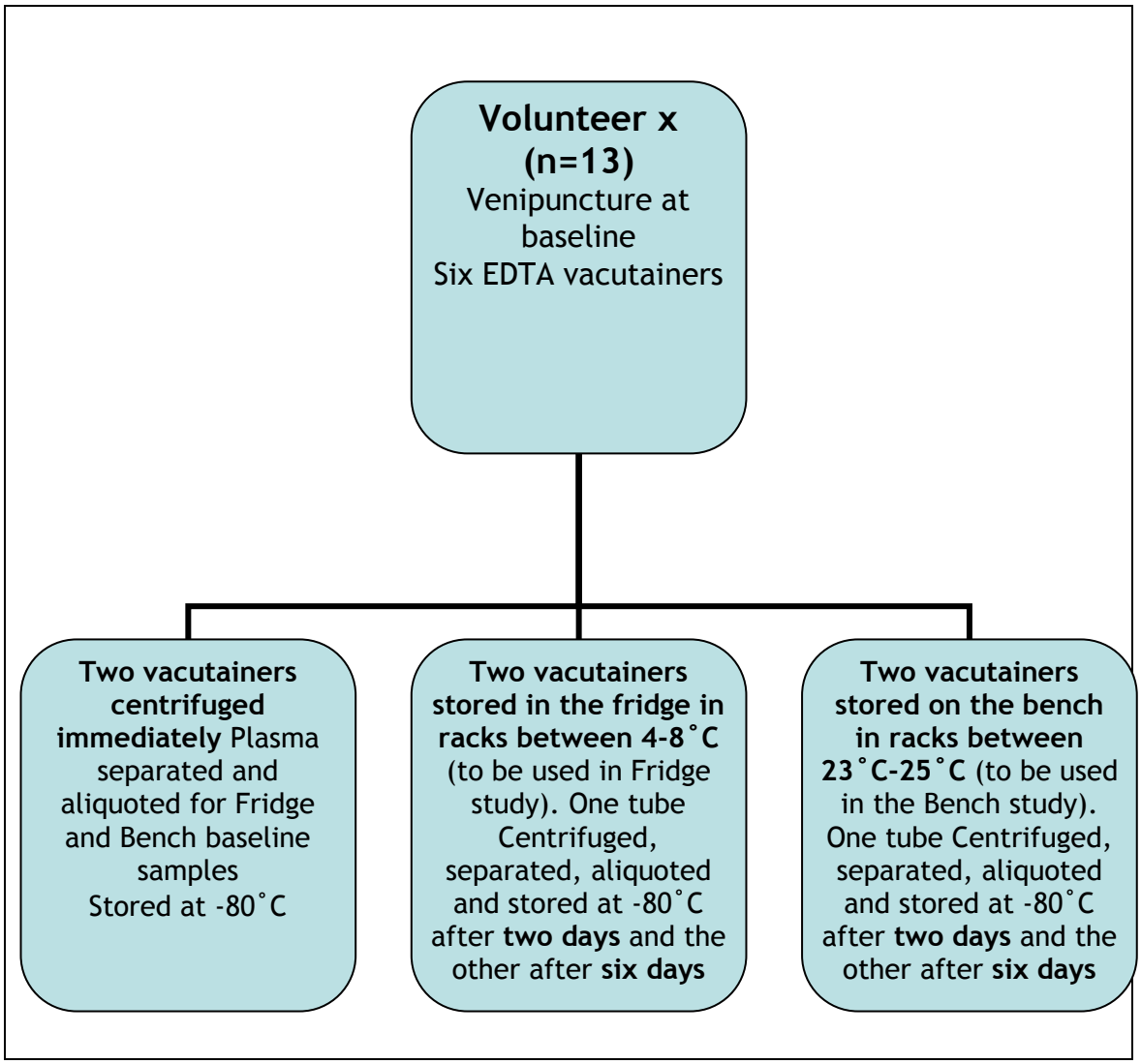
The aim of this study was to determine if leaving bloods un-separated (a) in a fridge/cold room (between 4-8˚C) and (b) on a bench at room temperature (23˚C - 25˚C) for up to 6 days after venipuncture would have an effect on measurable concentrations of total adiponectin in EDTA blood samples.

3.3.3 Study Subjects and Methods

Blood collection, storage and analysis

13 healthy volunteers, 8 female and 5 male (age 26 – 58 yrs), from the Vascular Biochemistry laboratory at the Glasgow Royal Infirmary were recruited to take part in the Fridge study and 10 people, 6 female and 4 male (age 26 – 58 yrs), from the same laboratory were recruited to take part in the Bench study (3 people were unable to be bled for all 6 tubes). Volunteers in the study signed an informed consent form (Appendix 5) giving permission for a trained phlebotomist to take blood. They were also informed that their bloods would be used for research purposes and stored on the 4th Floor of the Queen Elizabeth building at the Glasgow Royal Infirmary. On the day of venipuncture six 9mL EDTA vacutainers (BD Vacutainer Systems: Becton, Dickinson and Company) were collected from each volunteer. Not all of the volunteers were fasted. All samples were labelled with the sample ID, time and date of venipuncture, the study name (either Bench or Fridge study), and the time point (e.g. baseline, 2 days, 6 days). The cold room in our laboratory was kept between 4˚C -8˚C for the duration of this study and the bench laboratory temperature ranged between 23˚C - 25˚C.

Figure 3.4: Flow Diagram Showing the Processing of Blood for the Fridge and Bench Studies.



For each volunteer where possible two of the samples were stored in the refrigerator in a safe place between 4-8˚C, two of the samples were stored on the bench at room temperature (23˚C - 25˚C) and the other two tubes of EDTA blood were spun and the plasma separated immediately after venipuncture (3000rpm at 4˚C for 10 mins) in the under bench centrifuge (Beckman Coulter, GS-6KR). The plasma was immediately aliquoted into eppendorf tubes (red eppendorfs for the bench study and green eppendorfs for the fridge study) (Figure 3.3). These plasma samples were labelled Bench study baseline and Fridge study baseline respectively and stored in a -80˚C freezer. Two days after venipuncture another two EDTA blood tubes (one from the fridge and the other from the bench) for each volunteer were centrifuged and the plasma was aliquoted into labelled eppendorfs (volunteer ID, appropriate study name, 2 day sample) and stored alongside the baseline plasma samples in the -80˚C freezer. Finally six days after venipuncture, the last EDTA blood tubes from each volunteer (1 from the fridge and the other from the bench-top) were centrifuged and aliquoted into labelled eppendorfs (volunteer ID, study name, 6 day sample) to be stored alongside the other two samples in the -80˚C freezer. All three samples per person in each study were tested for total adiponectin concentrations using an R&D Systems Adiponectin ELISA kit (General Methods Section 2.4.1.1). All available data was analysed.

Statistical Analysis

Medians, 25th percentiles and 75th percentiles were calculated using an Excel spreadsheet. Dr. Paul Welsh advised on calculating out P- values and correlation coefficients. The Wilcoxon Matched-Pairs Signed-Ranks Test (Chapter 2) was used to establish p-values and the Spearman's rank Correlation Test (Chapter 2) was used to establish ρ -values. The website for IFA statistics was used for these tests (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on the book by Fleiss in 1986.

3.3.4 Results

Results from this study are summarised in tables **3.6 – 3.9**

Table 3.6 shows medians, and IQR's for the data obtained during the Fridge (between 4-8˚C) and Bench (25˚C) studies.

Table 3.7 summarises statistical analysis from the Fridge study where samples were left un-separated as whole blood for up to 6 days at 7˚C in a cold room before being processed. Comparisons between the baseline samples and those blood samples that were processed after being left for 2 days (48hrs) and 6 days (144hrs) in the fridge show no significant change in measurable concentrations of total adiponectin ($p= 0.54$ and $p=1$ respectively). There exists a significant correlation ($\rho = 0.87$, $p < 0.0001$ (48hrs); $\rho = 0.91$, $p < 0.0001$ (144hrs)) between the baseline sample values and the values obtained from samples that were left un-spun as whole blood for 2 days and 6 days in the fridge.

Table 3.8 summarises statistical analysis from the Bench study where samples were left un-separated as whole blood for up to 6 days on the bench-top between 23˚C-25˚C before being processed. Comparisons between the baseline samples and those blood samples that were processed after being left for 2 days (48hrs) and 6 days (144hrs) on the bench-top show no

significant change in concentrations of total adiponectin ($p=0.77$ for both time points). The 48 hour and 144 hour samples show a good correlation to the baseline samples ($\rho = 0.95$, $p < 0.0001$ for both time points).

Table 3.9 illustrates that there was no significant difference in measurable concentrations of total adiponectin in EDTA blood samples when left between 4-8˚C or at 25˚C and this trend was carried through for both 2 day old samples as well as those processed after 6 days ($p<=1$, 2 day samples; $p<= 0.16$, 6 day samples). Results obtained from samples left on the bench-top at 23˚C-25˚C correlate well with results from samples left in the fridge between 4-8˚C at both time points ($\rho = 0.90$, 48hr samples; $\rho = 0.92$, 144hr samples, $p<=0.0008$).

Figure 3.5 shows total adiponectin values in each subject at each time point (baseline, 48 hours and 144 hours) from the Fridge and Bench studies.

Table 3.6: Blood Sample Processing Results for Total Adiponectin

Means and SD's for all subjects

	Median (µg/mL)
Baseline (0hr)	9.92 **IQR** (5.93-12.98)
Fridge 2 days after venipuncture **(48hr at fridge temp.)**	9.77 **IQR** (7.82-11.04)
Fridge 6 days after venipuncture **(144hr at fridge temp.)**	9.41 **IQR** (6.22-12.05)
Bench 2 days after venipuncture **(48hr at bench temp.)**	8.75 **IQR** (5.01- 11.86)
Bench 6 days after venipuncture **(144hr at bench temp.)**	8.60 **IQR** (4.18-11.03)

Abbreviations for table 3.6: IQR, Inter-quartile range.

Table 3.7: Summary of Data From the Fridge Study Where Blood Samples Were Left Between 4-8˚C for 2 and 6 Days Un-separated as Whole Blood

	Baseline vs. 2days after venipuncture	Baseline vs. 6days after venipuncture
Wilcoxon matched-pairs signed-ranks test	p = 0.54	p = 0.99
Spearman's rank correlation coefficient	ρ = 0.87, p < 0.0001	ρ= 0.91, p < 0.0001

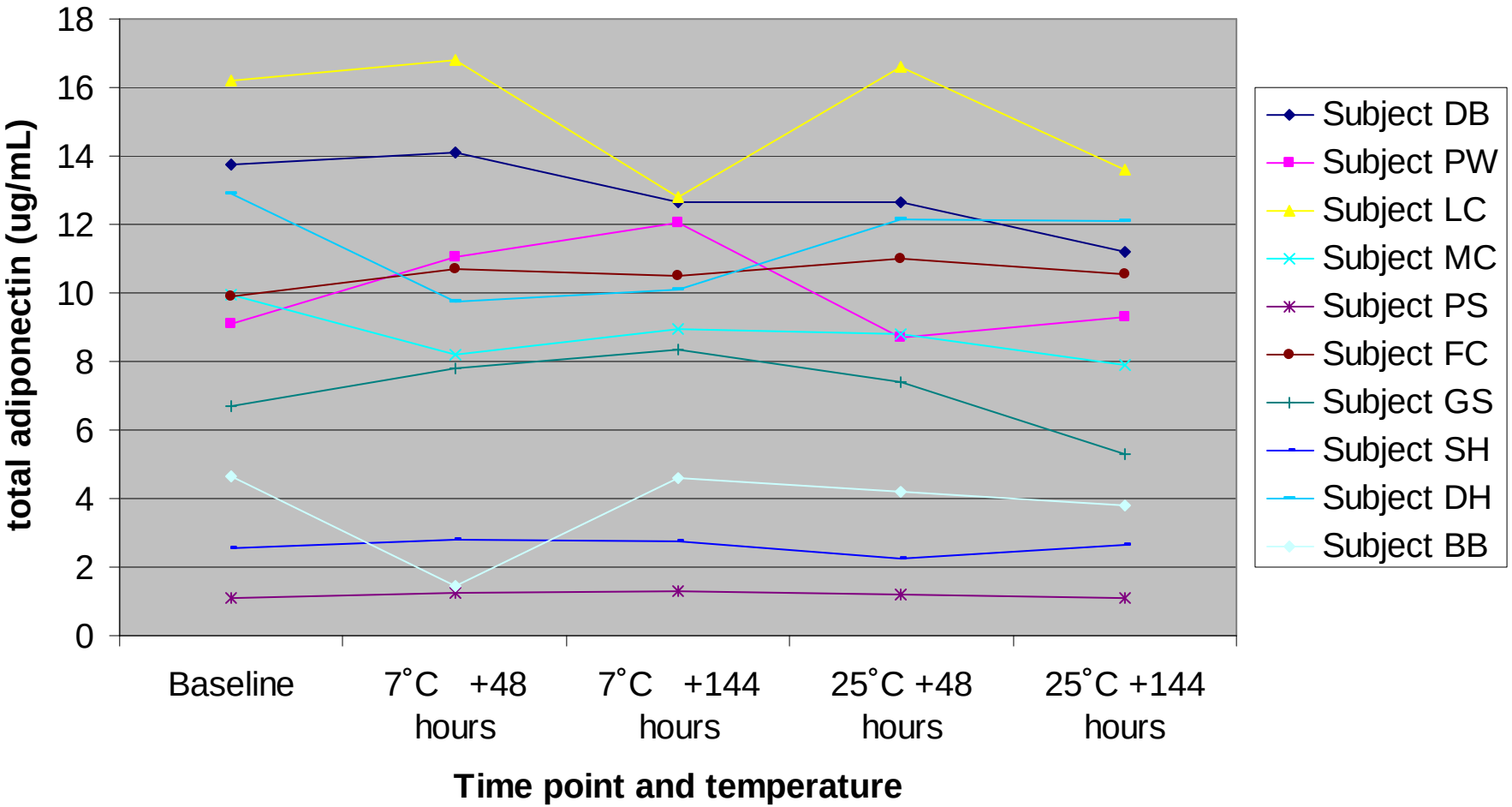
Table 3.8: Summary of Data From the Bench Study Where Blood Samples Were Left at Room Temperature (23˚C - 25˚C) for 2 Days and 6 Days Un-separated as Whole Blood

	Baseline vs. 2days after venipuncture	Baseline vs. 6days after venipuncture
Wilcoxon matched-pairs signed-ranks test	p = 0.77	p = 0.77
Spearman's rank correlation coefficient	ρ= 0.95, p < 0.0001	ρ= 0.95, p < 0.0001

Table 3.9: Comparison Between Data From the Bench Study With Data From the Fridge Study

	Bench vs. Fridge (2 days after venipuncture)	Bench vs. Fridge (6 days after venipuncture)
Wilcoxon matched-pairs signed-ranks test	p = 0.99	p = 0.16
Spearman's rank correlation coefficient	ρ= 0.90, p < 0.0001	ρ= 0.92, p < 0.0001

Figure 3.5 Blood Sample Processing



3.3.5 Discussion

Our findings show that blood samples left un-spun as whole blood in the fridge between 4-8˚C and on the bench at 23˚C -25˚C for up to 6 days are still viable for total adiponectin analysis and show no deterioration in this variable (table 3.7 and 3.8).

Importantly, when the Bench Study values were compared with the Fridge Study values for each time point (table 3.9), there was no significant change in the values and a good significant correlation between the data sets was obtained.

The results presented here found that measurable concentrations of total adiponectin were not significantly different in those specimens analysed after 48 hours or 144 hours compared with those that were processed immediately ($p = 0.99$) (table 3.9). These findings are in line with those reported by Pischon et al in 2003, where their findings report no significant change in adiponectin levels in 6 male and 6 female EDTA plasma samples when processed up to 36 hours after venipuncture (gender adjusted).

The findings here also extend to show that temperature did not seem to have an effect on measurable concentrations of adiponectin in these un-separated blood samples for up to 6 days.

3.4 Biological Variability

3.4.1 Introduction

Measurements of laboratory analytes for individuals are subject to many sources of variation. These include biological variation, pre-analytical variation (specimen collection), analytical variation (bias and imprecision), and post-analytical variation (reporting of results). Biological variation, the natural fluctuation of body fluid constituents around the homeostatic setting point, has two components: within and between-subject variation (Ricos et al, 2000). These components of biological variation are used to set analytical quality specifications for bias and imprecision, evaluate serial changes in individual analytes, and assess the clinical utility of population-based reference intervals (Lacher et al, 2005). Concentrations of a molecule in the blood can vary over the time of day, between seasons, can depend on whether the sample is fasted or non-fasted or can vary over a period much longer than a day depending on the activity of the subject. Sometimes variation in circulating concentrations of molecules can be caused by predictable external factors. These include eating patterns, activity and seasonal weather changes, thus allowing prediction of biological variation at a population level (Welsh, PhD, 2008). However it is those less predictable fluctuations that may be of more use in epidemiological studies, because they may provide insight into the usefulness of the molecule to predict disease. These include response to clinical infection, medication or as a response to rises in any other risk markers of disease. It is important to take biological variation into account when looking at risk associations in study cohorts. The same blood sample for each volunteer should be used to analyse all risk markers that can be measured using that type of sample. This will rule out potential fluctuating concentrations of variables in the blood that may confound the results if measured using different aliquots from the same subject.

In 1985, Ernst et al conducted a study involving 15 males and 10 females with a mean age of 23 ± 2. This study aimed to investigate intra-person variability over time. Blood was taken from the subjects on 4 separate occasions at

intervals of 7± 2 days. They assayed samples for plasma viscosity, hematocrit, red blood cell aggregation, red cell filterability and leukocyte count. After analysis they used the mean of the CVs and methodological CVs to calculate out the biological CV using the formula listed in methods below. Their results state that biological variation tends to be larger than methodological variation.

There is limited literature pertaining to the biological variation of adiponectin over time. In 2003, Pischon et al published a short technical brief on the stability of adiponectin in plasma samples and it's within person variation over one year. The pilot study to test for the 1 year reproducibility of adiponectin employed 300 randomly selected men from the previously mentioned (Chapter 1) Health Professional Follow up Study (HPFS) (Pischon et al, 2004). They included additional samples to assess the intra assay variation and found a CV of 20% (n=2). They adjusted their results for BMI, and found no significant difference (p=0.09) in adiponectin concentrations taken 1 year apart, but showed a high degree of reproducibility (intra-class correlation coefficient, 0.84, 95% Confidence Interval, 0.65-0.94). Therefore, they concluded by suggesting that a single measurement of adiponectin may be sufficient for risk assessment in epidemiological studies.

In a recently reported biological variation investigation, HDL-C was reported to have a dilution regression ration of 0.71 (The Emerging Risk Factors Collaboration, 2009). Since adiponectin has known associations with HDL-C, it is important to investigate whether the concentrations of this molecule fluctuate significantly.

3.4.2 Principle

The aim of this study was to investigate biological variation of total adiponectin by assessing whether total adiponectin levels vary significantly in an individual over the period of 6 weeks. This will allow us to determine if a single measurement of total adiponectin is sufficient for use in epidemiological studies.

3.4.3 Study Subjects and Methods

Blood collection, storage and analysis

9 volunteers, 8 women and 1 male (26 – 58 yrs), from the Vascular Biochemistry laboratory at the Glasgow Royal Infirmary were recruited for this study. Participants in the study signed an informed consent form (Appendix 5) giving permission for a trained phlebotomist to take blood. They were also informed that their bloods would be used for research purposes and stored on the 4th Floor of the Queen Elizabeth building at the Glasgow Royal Infirmary. None of the volunteers were smokers. One 9mL EDTA vacutainer (BD Vacutainer Systems: Becton, Dickinson and Company) was taken off each volunteer at the same time, on the same day each week, for six consecutive weeks. Volunteers were in the same fed state each week. Fasted samples are demarcated with an asterisk in table 3.12 below. After venipuncture the blood samples were centrifuged (3000rpm, 4˚C, 10 mins.) within 30 min. in an under-bench centrifuge (Beckman Coulter, GS-6KR). Plasma was separated and aliquoted into eppendorf tubes (2 x 1.5mL), using plastic pastettes. The eppendorf tubes were clearly labelled with sample ID, name of study and week, date and time of venipuncture. The plasma was then stored in the -80˚C freezer until analysis could be carried out. On the day of analysis all samples were snap-thawed at 37˚C, and all six samples per person were assayed on the same ELISA plate, to minimise inter-assay variation of results. Samples were analysed on the first defrost using the R&D Systems ELISA method (General Methods section 2.4.1.1). All available data was analysed.

Statistical analysis

Medians, SDs and CVs were calculated using an Excel spreadsheet.

The laboratory intra-assay variability (**table 3.10**) was quantified by running pooled EDTA plasma (from healthy volunteers) on an R&D systems ELISA assay (General Methods section 2.4.1.1). Three dilutions from the same plasma sample were prepared according to the R&D ELISA SOP and run 8 times on the same assay, thereby yielding 24 results in all. The means, standard deviations (SD's) and CVs from each dilution are calculated and tabulated below. The mean of the CVs was calculated using excel and used as the intra-assay CV in this study.

Laboratory inter-assay variability (**table 3.11**) was also quantified. As previously explained in chapter 2, this was done by running the same EDTA pooled plasma mentioned above, on 22 separate R&D systems total adiponectin ELISA assays (General Methods section 2.4.1.1) in duplicate. Means were calculated for each assay and then the mean of these means and standard deviations were used to calculate out a CV. As expected the inter-assay CV was higher than the intra-assay CV.

Total within person variation (Total CV) is the combined effect of biological CV and methodological CV (intra-assay CV), the latter being a constant value. Biological CV is therefore calculated using the following equation:

$$\text{Biological CV} = \frac{\text{Total CV-Methodological CV}}{\text{1-Total CV}}$$

(Ersnt et al, 1985)

(Refer to appendix 2 for full calculation)

Dr. Paul Welsh advised on statistical analysis. The Spearman's rank correlation test (Chapter 2, General Methods) was used to compare the concentrations of adiponectin taken at baseline to those taken in week 3, and then again comparing the baseline concentrations with those from week 6 (Table 3.14). Subject EM was unable to complete the study and so comparisons for baseline/week 6 use data from only 8 samples for calculation purposes. The website for IFA statistics was used for these tests (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on those in the paper by Ernst et al, 1985.

The intra-class correlation coefficients (Chapter 2, General Methods) were calculated out according to the book on "the design and analysis of clinical experiments" by Fleiss, 1986, using an excel spreadsheet. I received guidance from Dr. Paul Welsh when performing these tests.

Table 3.10: Laboratory Intra-Assay Variability

Sample	n	Mean (µg/mL)	SD	CV (%)
A	8	14.50	0.65	4.50
B	8	15.56	0.44	2.85
C	8	17.06	0.83	4.86

Abbreviations for Table 3.10: SD, Standard deviation; CV, coefficient of variation.

For the calculation of biological CV, using the equation on the previous page, the mean of these CVs was used as the methodological CV.

This was calculated on an excel spreadsheet as **4.07 %.**

See appendix for full calculation.

Table 3.11. Laboratory Inter-Assay Variability

	n	mean	SD	CV (%)
Adiponectin (µg/mL)	22	15.64	1.05	6.73

Abbreviations for Table 3.11: SD, Standard deviation; CV, coefficient of variation.

3.4.4 Results

The effects of short term biological variation on adiponectin concentrations in plasma are summarised in **Tables 3.12 - 3.15**
Blood samples were taken from 9 healthy volunteers and there were 50 time points in total. Subjects DB and EM were unable to give all 6 time points. All available data was analysed.

Table 3.12 shows the medians, 25th percentiles and 75th percentiles from six weeks of results in all 9 volunteers.

Table 3.13 shows the total variability in the 9 volunteers that took part in the study. Total CV comprises both a methodological CV and biological CV component and these have been calculated out accordingly using the formula above and tabulated. As expected the biological CV was greater than the methodological CV, and the mean of intra person means falls within the normal range adiponectin concentration value for healthy individuals (0.5-30 ug/mL) (Magkos et al, 2007).

As we can see from the Spearman's Rank Correlation coefficients reported in **table 3.14**, total adiponectin concentrations correlate with one another throughout the six weeks.

Table 3.15 summarises the intra-class correlation coefficient and 95% confidence intervals for reliability measurements. The intra-class correlation coefficient is (0.99, 95% CI: 0.98 – 1.00).

Figure 3.6 shows changes in adiponectin concentrations in all subjects over six weeks.

Table 3.12: Intra Person Medians (and IQR) of Weekly Measurements of Adiponectin. Results Obtained from 50 Observations From 9 People (Subjects DB and EM Were Unable to Give all 6 Time Points.

	Median (µg/mL)
DB*	13.28 **IQR** (13.20 - 13.63)
PW*	9.93 **IQR** (8.84 - 11.52)
LC	15.45 **IQR** (14.74 - 17.21)
MC	9.11 **IQR** (8.69 - 9.95)
KU	6.64 **IQR** (5.84 -7.06)
PS*	1.10 **IQR** (1.05 - 1.07)
EM	11.59 **IQR** (10.96-12.06)
FC	10.70 **IQR** (10.18-11.11)
GS	5.78 **IQR** (5.39-6.12)

Abbreviations for Table 3.12: CV, coefficient of variation; IQR, Inter-quartile range.

Table 3.13: Total CV and the Relative Proportions That are Methodological (Intra-Assay) or Biological in Origin.

Results obtained from 50 observations from 9 people (subjects DB and EM were unable to give all 6 time points; refer to appendix for full table of results).

Measured Protein	Mean of intra-person means	Mean of intra-person SD's	Mean of intra-person CV's (%)	Methodological CV %	Biological CV %
Adiponectin(µg/mL)	9.37	1.21	12.90	4.07	10.14

Abbreviations for Table 3.13: SD, Standard deviation; CV, coefficient of variation.

Table 3.14: Spearman's Rank Correlations From the Comparison of Adiponectin Concentrations at Week 1 With Week 3 and Week 1 with Week 6.

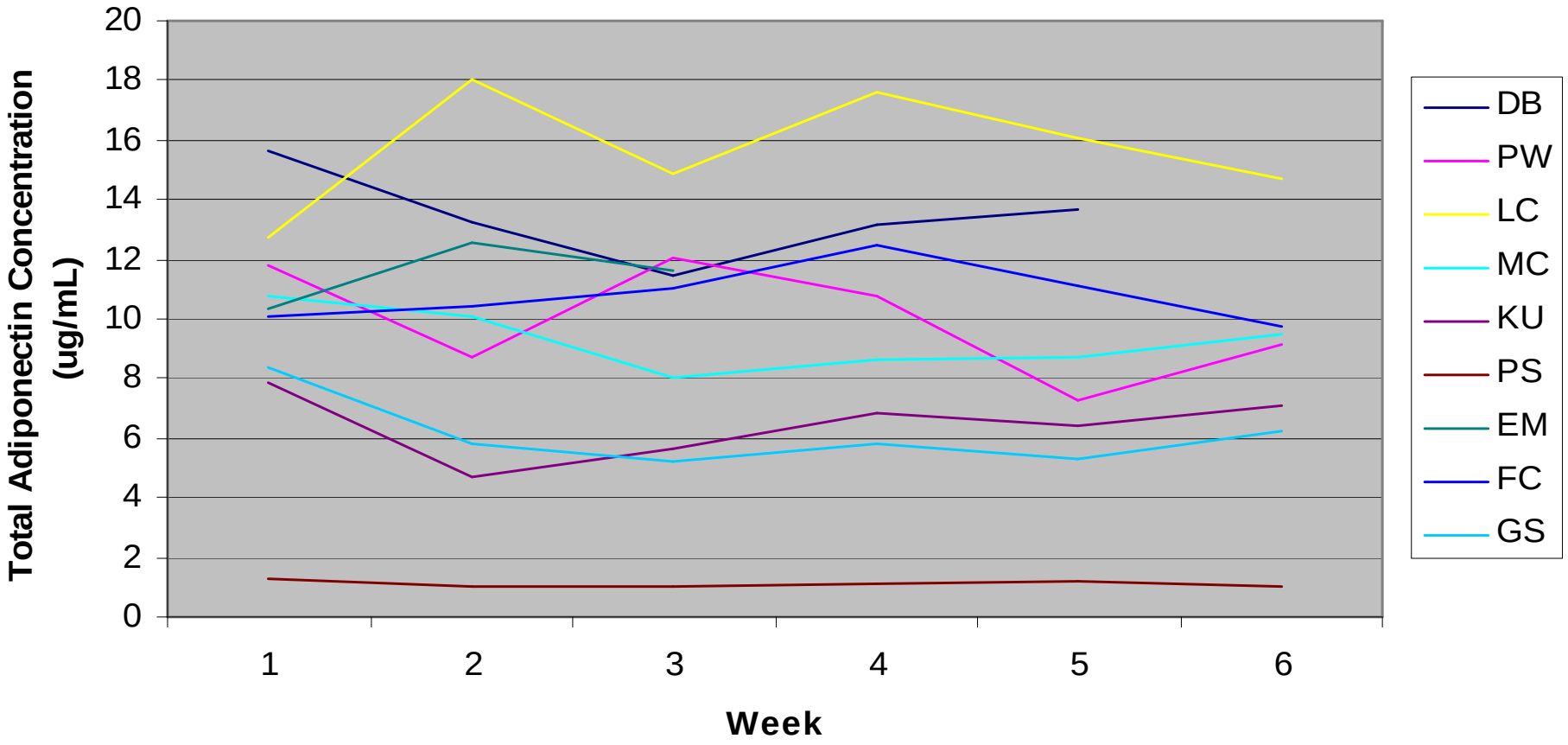
Data Compared	Spearman's rank correlation coefficient
Baseline with week 3	$\rho = 0.78$, $p <= 0.02$
Baseline with week 6	$\rho = 0.85$, $p <= 0.01$

Table 3.15: Intra-Class Correlation Coefficients For Reliability Measurements in Short Term Variation.

	Intra-class correlation coefficient (95% CI)	n required measurements for 75% reliability nrep (95% CI)	n required measurements for 90% reliability nrep (95% CI)
Adiponectin	0.99 (0.98 – 1.00)	1 (1-1)	1 (1-1)

Abbreviations for Table 3.15: CI, Confidence Interval; nrep, number of required repeat measurements.

Figure 3.6: Changes in Total Adiponectin Concentrations in Each Subject over Six Weeks



3.4.5 Discussion

It is important to consider the background biological variation in prospective studies. A single assessment of a biochemical indicator may be prone to short-term variation and can lead to under-estimation of risk association (Pischon et al, 2003). The danger of misclassifying an individual into a particular risk category, be it high, intermediate or low, increases with a decrease in reproducibility of a marker, therefore damagingly impacting on the usefulness of the marker as a clinical tool (Welsh, PhD, 2008).

In this study the intra-assay CV (4.07%), is in line with that given by industrial kit parameters (2.5 - 4.7%, R&D systems ELISA; <=3.0, Mercodia and 5.4%, ALPCO Diagnostics ELISA). Although, the mean intra person CV was relatively high (12.9%), the biological component of this was fairly low (10.14%) as compared to some of the inflammatory cytokines (55.7%, CRP and 50.1%, IL-6) (Welsh, PhD, 2008), suggesting adiponectin is fairly stable in healthy volunteers. Interestingly, taking into account the known positive correlations adiponectin has with HDL cholesterol, a meta-analysis of 30 studies published from 1970-1992 yielded estimates of biological CV of 7.4% for HDL cholesterol (Smith et al, 1993). Therefore our findings from this small study are broadly consistent with known percentages of variability in established risk markers (e.g. HDL-C) that display robust correlations with adiponectin.

The good correlations that this study reports between baseline and week 3 (ρ = 0.78) and between baseline and week 6 (ρ = 0.85) suggests that intra-individual adiponectin concentrations are fairly stable over short term periods (up to 6 weeks) (table 3.14). Also, the intra-class correlation coefficient (0.99, 95% CI: 0.98 – 1.00) (table 3.15) in this study shows that total adiponectin has a high degree of reproducibility and these findings, that a single measurement of adiponectin concentration is sufficient for risk assessment in epidemiological studies. These findings are in line with those from the 2003 study by Pischon et al.
Figure 3.5 shows no linear trend between mean adiponectin concentrations and CV. Therefore CV appears to be independent of the plasma level of adiponectin. Larger studies would give a more accurate depiction of this

relationship, but at face value, these data are reassuring as they suggest the adiponectin assay performs well across a range of values.

Limitations of the study

There were a few limitations to the study that merit discussion. The 50 samples analysed were not all in the same fed or fasted state. Only 3 of the volunteers were in a fasted state when the bloods were taken each week. However the state was consistent for each subject throughout the 6 weeks and from recent literature, it seems adiponectin levels do not alter postprandially (Caixas et al, 2006).

This study was not sufficiently large enough to stratify by sex, age and lifestyle factors. All, except two, women were pre-menopausal, and all individuals, as far as we can judge, led healthy lifestyles. Of note, the males had reduced levels of circulating adiponectin. Of course variation exists due to other external factors, for example genetic or other unknown metabolic or hormonal factors. However, it may not be possible to explain much more of the variation in adiponectin concentrations because of biological variation, as the mechanism for rises in adiponectin concentrations still requires further investigation. As it stands presently, adiponectin shows random pulsatility in its secretion (Punthakee et al, 2006). Other factors affecting the reproducibility of adiponectin should be considered, these include long term storage, freeze/thaw cycles and temperature during assay. Although, our prior studies in this thesis suggest adiponectin is in fact quite stable to many such considerations.

Also, due to time restraints we were unable to carry on the work for a longer period of time. We suggest that additional studies be carried out with larger volunteer numbers over a longer study time period and with all volunteers in the same fed state.

It may also be interesting to look at biological variability of other forms of adiponectin, particularly HMW adiponectin as it is thought to be the more biologically active form of the molecule.

Chapter IV: Methodological Considerations - Effects of Analytical Variables on Measurements of Adiponectin

4.1 Introduction to Analytical Variables

Analytical variables refer to problems or differences that may arise with performance of a test in the laboratory. The term 'analytical variable' refers to everything that affects a measurement: the method, the equipment, the reagents, the analyst, the laboratory environments, etc. Analytical variability can be reduced by adopting a stringent quality assurance procedure and by working to good laboratory practice (GLP) standards.

The first study in this chapter investigates compatibility of commercial ELISA kits available on the present market for the measurement of total adiponectin. It is important that the variability between commercial kits be considered when making comparisons between epidemiological studies, because if results obtained for total adiponectin are measured on different kits and are not comparable, then assumptions drawn from comparisons of the two data sets cannot be validated.

The second study in this chapter aims to validate the use of citrated plasma samples for the R&D Systems adiponectin ELISA kit. At present only EDTA plasma, serum and cell culture supernates have been validated for use on this kit. Sometimes a study may only have one type of aliquot obtainable from each volunteer/patient and it may be necessary to do all available tests on this sample if re-bleeding is not an option. This validation may add further scope for total adiponectin analysis when using this kit.

4.2 Analysing Citrated Samples for Total Adiponectin Using R&D ELISA Methodology

4.2.1 Introduction

Compounds that do not allow blood to clot are called anticoagulants. The most common of these include EDTA, Heparin and Citrate. Most clinical laboratory tests are performed using blood collected in EDTA or serum (Green et al, 2008). Situations may arise where blood has been drawn from a patient and preserved with only one type of anticoagulant and so all tests need to be performed on this sample, unless the patient is available for re-bleeding. All current ELISA assays recommend the use of human serum, EDTA plasma, or Li-Hep plasma for HMW adiponectin. In addition serum, plasma or cell culture supernates are recommended for total adiponectin analysis. It clearly states in the user guide for these assays that citrated plasma has not yet been validated for use in analysis.

Citrate is usually used for coagulation tests, as well as in blood transfusion bags. It gets rid of the calcium, but not as strongly as EDTA (wikipedia). It can be in the form of sodium citrate or Acid Citrate Dextrose Solution (ACD). Occasionally our laboratory receives only citrated plasma samples for a study. We generally like to measure as many variables on a sample as possible to gain a better understanding of disease and the associations between molecules in the pathogenicity of these various diseases, in particular CVD and Diabetes.

It is for this reason that this study aims to validate the use of citrated plasma samples in the total adiponectin ELISA assay. There is limited literature on this and so validation may prove to be a novel method for adiponectin analysis.

4.2.2 Principle

The aim of this study was to validate samples that have been preserved with citrate anticoagulant for use in a total adiponectin R&D systems ELISA assay (general methods chapter 2.4.1.1). We had a cohort from the Postman study[4] in which samples had both an EDTA and a citrate aliquot for each volunteer. We assayed 109 EDTA plasma samples and the same 109 volunteers' citrated plasma samples on an R&D systems ELISA assay and compared the results to see if there was a significant difference in values between the separate anticoagulant factors and whether the results rendered from the two sample types correlated.

4.2.3 Study Subjects and Methods

Blood collection, storage and analysis

The following participant recruitment has been previously described elsewhere (Chastin et al, 2009). Participants for the study were postal workers, recruited from offices in Glasgow, UK. Postal workers within Glasgow were informed of the study via work e-mail, and participants volunteered to take part in the study. Postal workers were selected because there were clear distinctions in required activity levels at work between occupational categories. In this study, participants were either delivery postal workers who walked their delivery route (active workers) or office-based postal workers (sedentary workers). These categories were used to allow a comparison between individuals with typically active and sedentary employment types. Participants were non-smokers aged between 20 and 60 years, had been working for at least 3 months prior to the study, and were not on any medication for chronic heart disease, diabetes, hypertension or hypercholesterolemia.

[4] Study looking at postal workers in the Glasgow area, UK. Postal workers have clear distinctions in required activity levels at work: desk/office job vs. postal delivery worker. This study allows comparison between individuals with active and sedentary employment and aims to look at how lifestyle affects CVD risk factors, diabetes, obesity and general well being.

Written informed consent was obtained from all participants. The study was approved by the School of Health and Social Care, Glasgow Caledonian University ethics committee, and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

I obtained consent from the Principle investigator of the Postman study (Dr. William Tigbe) to include my findings from his samples in my study.
Blood was drawn, by a trained medical practitioner, into two EDTA vacutainers (BD Vacutainer Systems: Becton, Dickinson and Company) and a citrate vacutainer (BD Vacutainer Systems: Becton, Dickinson and Company). These were placed on ice at the post office where blood was taken and immediately transported back to the Vascular Biochemistry laboratory for separation. The bloods were processed within 2 hours of venipuncture, where they were spun (3000rpm at 4˚C for 10 min.) in an under-bench centrifuge (Beckman Coulter, GS-6KR) and the plasma separated with pastettes and aliquoted into eppendorf tubes (Blue for citrate and red for EDTA). The eppendorfs were clearly labelled with sample ID, date and time of venipuncture and placed in a -80˚C freezer until analysis could be carried out on them. On the day of analysis samples were removed from the freezer in batches of 78, snap-thawed at 37˚C and assayed on a R&D systems ELISA assay (general methods, chapter 2.4.1.1). Since each plate can only accommodate 78 study samples and 2 controls, two plates were used to fit the 109 EDTA samples on for analysis and two separate plates were used to fit the 109 citrate samples on. Total adiponectin and citrate were measured on the first defrost of these samples. All available data was analysed.

Statistical Analysis

Medians and CVs were calculated using an Excel spreadsheet. Dr. Paul Welsh advised on calculating out p- values and correlation coefficients. The Wilcoxon Matched-Pairs Signed-Ranks Test (Chapter 2) was used to establish p-values and the Spearman's rank Correlation Test (Chapter 2) was used to establish ρ -values. The website for IFA statistics was used for these tests (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on the book by Fleiss in 1986.

4.2.4 Results

Table 4.1 summarises medians, 25th percentiles and 75th percentiles (IQR's) for both the citrate and EDTA sample types in all 109 volunteers.

Table 4.2 shows results from statistical analysis on the two sample types. There seems to be a significant change in concentrations of total adiponectin when measured on citrate samples compared to concentrations obtained from the EDTA samples ($p< 0.001$) (both measured using the R&D systems ELISA method). The Spearman's Rank Correlation Coefficient between these two data sets was 0.96 and highly significant ($p< 0.001$).

Figure 4.1 illustrates a Bland Altman plot of the mean of EDTA and Citrate log-transformed values vs. the difference between the log-transformed values of adiponectin concentrations from each sample type (Citrate and EDTA) using the R&D systems ELISA assay (general methods, chapter 2.4.1.1). Citrate values seem to be consistently lower than the EDTA values obtained using this assay.

Table 4.1 Summary of Data for EDTA Plasma and Citrate Plasma Measured on an R&D ELISA Assay for the Determination of Human Total Adiponectin

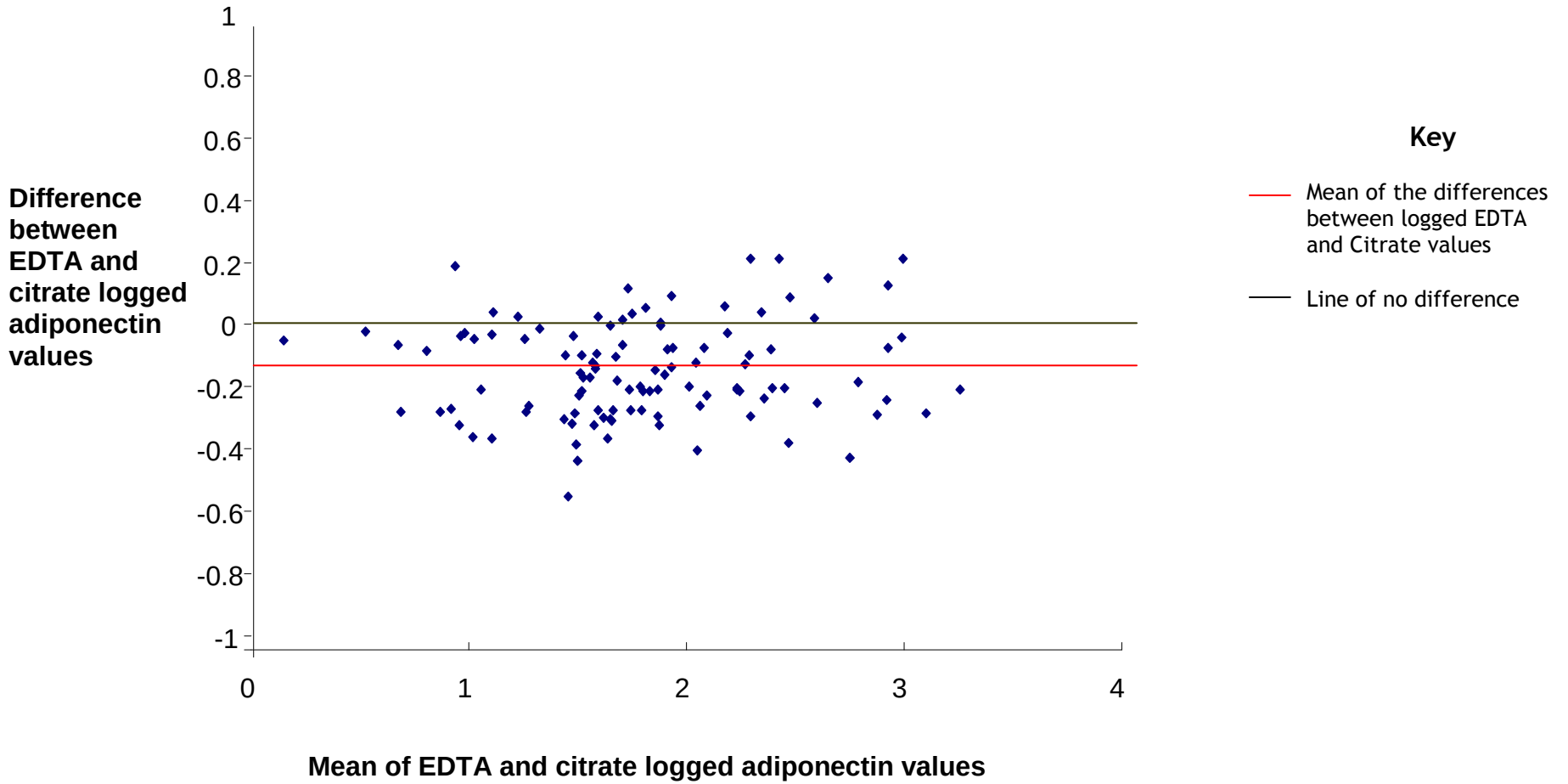
Type of sample	Median (µg/mL)
EDTA Plasma	6.10 **IQR** (4.89 - 9.47)
Citrate Plasma	5.34 **IQR** (3.84 - 8.48)

Abbreviations for table 4.1: IQR, Inter-quartile range.

Table 4.2 Summary of Data from the Comparison of Total Adiponectin Results Obtained from Citrate Plasma and EDTA Plasma

Statistical test	Citrate Plasma vs. EDTA Plasma
Wilcoxon matched-pairs signed-ranks test	$p < 0.0001$
Spearman's rank correlation coefficient	$\rho = 0.96$, $p < 0.0001$

Figure 4.1: Illustrating Bland Altman Plot of Mean of EDTA and Citrate Logged Total Adiponectin Values vs. the Difference between the EDTA and Citrate Logged Adiponectin Values



4.2.5 Discussion

It is imperative to know the viability of sample types for different laboratory tests. In most cases the sample types that have been validated for the assay will be stated in the assay SOP. Although EDTA plasma is the most widely used sample type for most laboratory analysis, it may be necessary in some instances for another type of sample to be used. As is the case with large epidemiological studies, and if finance permits, a researcher would aim to test as many variables on that **one** sample to get a wider knowledge on the interactions of molecules in a disease/healthy state.

In this study one aim was to validate Citrate plasma samples for use on an R&D total adiponectin ELISA assay. From statistical analysis of the data (table 4.2), we can see that although there may be a significant difference in the actual concentrations of total adiponectin between the citrate and the EDTA samples ($p < 0.0001$), the highly significant correlation coefficient of 0.96 demonstrates that the samples maintain rank order between the two sample types. Figure 4.1 shows a Bland Altman plot of the mean of the EDTA and citrate samples vs. the difference between the two measurable concentrations from each sample type. The plot shows that on average the Citrate samples rendered lower total adiponectin values than the EDTA samples did (those values below the red line) (Figure 4.1). Looking at absolute concentrations, the total adiponectin concentrations measured on citrate samples were about ~1µg/mL lower than those concentrations obtained from the EDTA samples. This discrepancy is almost certainly due to the dilution effects, since citrated tubes contain 0.5mls of fluid to which 4.5mls of blood is added.

In conclusion, EDTA samples produce higher total adiponectin values than the citrated samples do when using this ELISA assay, but the correlation is still very good. Therefore citrated plasma samples could be validated for use. It's not an issue that the citrate values are slightly lower, as long as they are consistently lower within a study. However, it must be noted that the same type of sample should be used throughout the duration of a study; study

results would not be comparable if one half of the cohort was measured on EDTA plasma and the other half of a cohort on citrate plasma.

We suggest that larger studies are needed to corroborate this result.

4.3 Comparison of Total Adiponectin Measurement by Three Different Commercially Available ELISA Kits

4.3.1 Introduction

As previously mentioned, adiponectin automatically self-binds to form larger structures and there are different multimeric forms including LMW trimers, MMW hexamers, HMW oligomeric structures and finally globular adiponectin. The varying forms have altered biological activity and therefore may also have separate functions.

A human adiponectin ELISA is designed to measure the concentration of molecules in human plasma/serum. Until recently ELISA's for the determination of adiponectin allowed only for the measurement of total adiponectin on samples. However, interest in adiponectin multimerisation and the regulation of its isoform distribution was fuelled by several studies indicating that most of the biological activity of adiponectin was attributed to the HMW form rather than to the LMW and MMW complexes. It became necessary for there to be a method capable of measuring all of the multimeric complexes. A handful of methods have been used for the isolation and measurement of HMW adiponectin, based on velocity gradient centrifugation (Scherer et al, 1995), gel filtration chromatography (Tsao et al, 2002) and polyacrylamide gel electrophoresis (Waki et al, 2003). However more recently, novel ELISA's have been developed to measure not only the total adiponectin concentrations in a given sample, but also the varying oligomeric forms of this molecule.

Prior to the introduction of this novel kit from ALPCO diagnostics (General Methods section 2.4.2), only total adiponectin measurement was available, the most popular of these being the R&D Systems adiponectin ELISA (general Methods 2.4.1.1).The R&D kit makes use of antibodies raised against human adiponectin and so allows for the direct determination of plasma levels of the protein. The ALPCO kit still employs the same sandwich enzyme technique typical of the ELISA assay, but also allows for the measurement of all of the multimeric complexes by adopting selective proteases to digest the molecule

into its varying molecular weights. Also reported in this study is the performance of the Mercodia ELISA assay for the determination of total adiponectin concentrations in human samples (general methods 2.4.1.2). It is a new addition to this line of ELISA assays. It employs the same sandwich enzyme technique as the other two assays in which two monoclonal antibodies are directed against separate antigenic determinants on the adiponectin molecule, but like the R&D systems product only allows for the determination of total adiponectin concentrations.

It has become common practice (if finance allows) for researchers in this field to measure both the total and HMW species and to express these as a ratio to one another. The advantage of the ALPCO ELISA method is the ability to measure total concentrations of the molecule as well as the various forms of adiponectin by utilising the same standards and the same protocol. Therefore one is able to determine a more realistic analysis of the adiponectin distribution in a given sample. This is a more reliable way of determination as opposed to using one method for measuring total adiponectin and another for HMW adiponectin measurement. This is important as some studies (Magkos et al, 1995; Bluher et al, 2007) report differences in the ability of commercially available assays for total adiponectin. This is likely due to the variable specificity of the different antibodies used for each adiponectin isoform (Magkos et al, 1995). However, at times it may be the case that total adiponectin has already been measured on a data set in the past, with a kit, that at the time, only allowed for the measurement of total adiponectin, and as it has become common practice to now include a ratio incorporating the HMW adiponectin measurement, a different kit now needs to be employed for this purpose. Therefore we need to determine whether these two kits correlate to one another.

By comparing the total adiponectin concentrations obtained from the ALPCO and Mercodia kits to the adiponectin concentrations rendered from the R&D Systems kit, we could examine whether the kits correlated and whether the three kits produced similar results.

4.3.2 Principle

We had a sample set from the EarlyBird[5] Cohort (Murphy et al, 2008) which had previously been analysed for total adiponectin in the original cohort, using the R&D Systems ELISA method. When the ALPCO and Mercodia kits became available we measured total adiponectin on the same sample set using these kits.

We measured total adiponectin on a subset of 75 serum samples from the EarlyBird Cohort, using the ALPCO diagnostics ELISA method and the Mercodia adiponectin ELISA method. Results from these tests were then compared to the previously determined total adiponectin concentrations measured using the R&D Systems ELISA method.

[5] The EarlyBird study involves 300 schoolchildren and their families. It is a 12-year diabetes study to find which factors in childhood lead to the development of diabetes later in life. The study gathered unique data on the factors that underlie the early development of diabetes/heart disease (physical activity, energy expenditure, dietary choices and body composition), the influence of those factors on the insulin resistance that underlies disease and the impact of insulin resistance on the markers of disease as children grow and mature (http://www.earlybirddiabetes.org/

4.3.3 Study Subjects and Methods

Blood collection, storage and analysis

The collection of these samples started in 2000 and continued right up until 2008. The method for blood collection has been documented elsewhere (Murphy et al, 2008). In short, children attended a hospital-based outpatient facility at 0800–0900 after an overnight fast of at least 10 hours. Venous blood was collected into Vacutainer Tubes (BD Vacutainer Systems: Becton, Dickinson and Company) and delivered to the laboratory within 2 hours. After centrifugation (1500g for 10 min), samples for the original Earlybird cohort study were either analysed straight away or stored at -20˚C. When samples were transferred to our laboratory they were stored at -80˚C.

For the purpose of this study and due to financial constraints (high price of ALPCO kits), eighty samples were chosen randomly from the Earlybird cohort study. This set consisted of samples from 40 volunteers each with two visits. However not all volunteers were able to attend both visits, therefore there were 75 samples available for analysis. Total adiponectin concentrations were measured using the:

1. R&D systems ELISA method (General Methods 2.4.1.1)
2. Mercodia adiponectin ELISA method (General Methods 2.4.1.2)
3. ALPCO diagnostics ELISA method (General Methods section 2.4.2).

All available data was analysed.

Statistical analysis

Medians were calculated using an Excel spreadsheet. Dr. Paul Welsh advised on calculating out P- values and correlation coefficients. The Wilcoxon Matched-Pairs Signed-Ranks Test (Chapter 2) was used to establish p-values and the Spearman's rank Correlation Test (Chapter 2) was used to establish ρ -values. The website for IFA statistics was used for these tests (http://www.fon.hum.uva.nl/Service/Statistics.html). Calculations were based on the book by Fleiss in 1986.

4.3.4 Results

Table 4.3 illustrates medians, 25th percentiles and 75th percentiles for total adiponectin measurements on the three kits (R&D systems, Mercodia and ALPCO Diagnostics). On average the concentrations of total adiponectin measured on the ALPCO kit were lower than when measured with the other two kits.

Table 4.4 summarises statistical analysis of comparisons between the R&D systems ELISA and the ALPCO Diagnostics ELISA. There is a highly significant difference between total adiponectin concentrations measured in the same set of samples on the R&D kit and those obtained from the ALPCO Diagnostics kit ($p< 0.0001$). The data set rendered a correlation coefficient of 0.89 and this was a highly significant correlation ($p< 0.0001$).

Table 4.5 summarises statistical analysis of comparisons between the R&D systems ELISA and the Mercodia ELISA method. There is a highly significant difference between total adiponectin concentrations measured in the same set of samples on the R&D kit and those obtained from the Mercodia kit ($p< 0.0001$). The data set rendered a correlation coefficient of 0.83 and this was a highly significant correlation ($p< 0.0001$).

Figure 4.2 illustrates a Bland Altman plot of the means of the log-transformed concentrations measured on the R&D and ALPCO kits vs. the difference between the log-transformed concentrations rendered from each of these kits. There is a suggestion of a slight negative bias such that levels are proportionately lower as adiponectin concentrations increase.

Figure 4.3 illustrates a Bland Altman plot of the means of the log-transformed concentrations measured on the R&D and Mercodia kits vs.

the difference between the log-transformed concentrations rendered from each of these kits.

Table 4.3: Summary of Data from Total Adiponectin Concentrations Measured on 80 Samples With the R&D Systems ELISA Assay, the ALPCO Diagnostics ELISA Assay and the Mercodia ELISA Assay.

	Median (µg/mL)
R&D Systems	10.48 **IQR** (8.41-13.89)
Alpco Diagnostics	7.37 **IQR** (6.27-9.08)
Mercodia	9.86 **IQR** (8.17-12.07)

Abbreviations for table 4.3: IQR, Inter-quartile range.

Table 4.4: Comparison of Total Adiponectin Measurements Taken From the R&D Systems Kit and the ALPCO Diagnostics Kit.

	R&D Systems vs. ALPCO
Wilcoxon matched-pairs signed-ranks test	$p < 0.0001$
Spearman's rank correlation coefficient	$\rho = 0.89$, $p < 0.0001$

Table 4.5: Comparison of Total Adiponectin Measurements Taken From the R&D Systems Kit and the Mercodia Kit.

	R&D Systems vs. Mercodia
Wilcoxon matched-pairs signed-ranks test	$p < 0.0001$
Spearman's rank correlation coefficient	$\rho = 0.83$, $p < 0.0001$

Figure 4.2: Bland Altman Plot Illustrating the Means of the Logged Adiponectin Values from the R&D and ALPCO Kits vs. the Difference Between the Logged Adiponectin Values Rendered from Each Kit

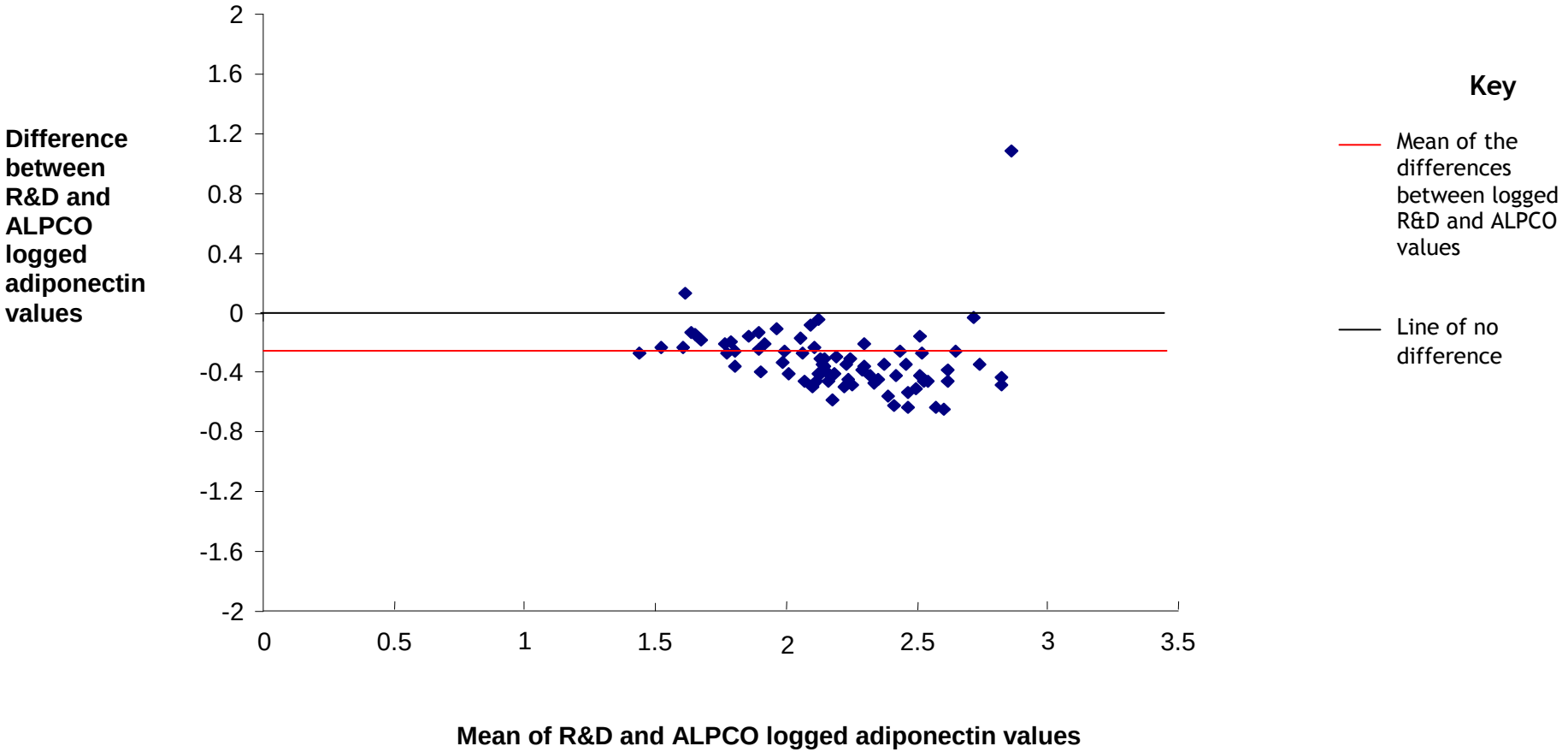
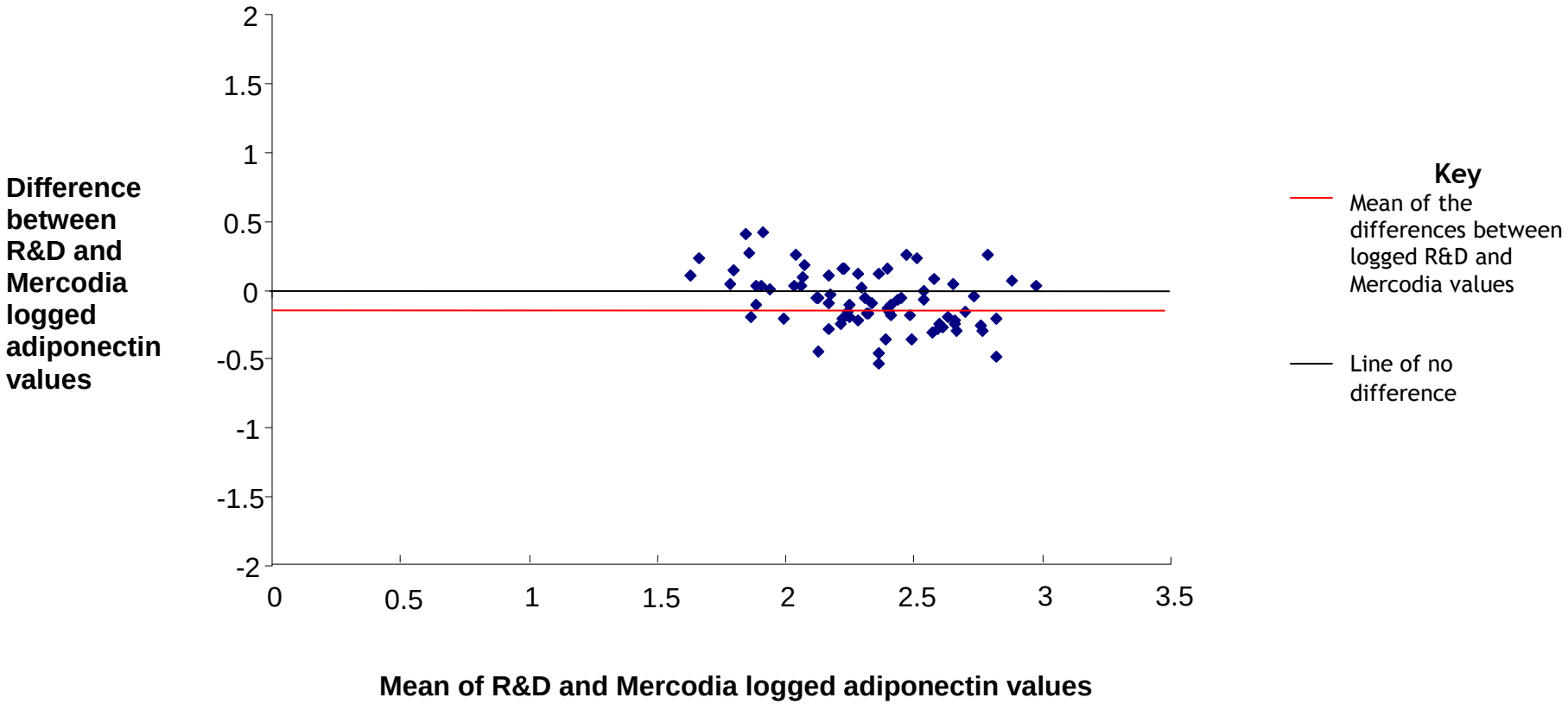


Figure 4.3: Bland Altman Plot Illustrating the Means of the Logged Adiponectin Values from the R&D and Mercodia Kits vs. the Difference between the Logged Adiponectin Values Rendered from Each Kit



4.3.5 Discussion

In order to make comparisons between large epidemiological studies, researchers need to be sure that the different commercial kits available for analyte measurement are comparable to one another. This study tested three commercially available ELISA kits for the determination of human adiponectin. Two of the kits (R&D and Mercodia) allow only for the measurement of total adiponectin and the third kit (ALPCO diagnostics) allows for the determination of all forms of the molecule, by use of selective proteases. The performance of the ALPCO and R&D kits from this study has been published in the Journal of Clinical Endocrinology & Metabolism (Sattar et al, 2008) and presented in chapter 5 of this thesis.

In the comparison between all of the kits there are highly significant differences in the concentrations of total adiponectin measured. On average, the R&D systems kit renders the highest values (8.41-13.89) followed closely by the Mercodia kit (8.17-12.07) and the ALPCO diagnostics kit (6.27-9.08) giving the lowest values. This may be due, in part, to the extremely high sample dilution factor (1:5151) used in the ALPCO methodology compared to the R&D (1:100) and the Mercodia (1:101) kits. There is more chance for error in the ALPCO methodology too, as the dilution requires two dilution steps whereas the other two methods described in chapter 2 only require a single dilution step. This may also explain the slight negative bias seen in figure 4.2, where at higher levels of adiponectin concentrations, the difference in values between the kits is proportionately larger. With this noted however, it is reassuring to report that the ALPCO and R&D kits were as closely ranked (ρ =0.89) as the R&D and Mercodia kits (ρ =0.83). The very strong correlation coefficient factors suggest that there is a very good correlation between all kits, and although there may be differences in

the absolute concentrations, as long as this difference is consistent, comparisons between kits is good. It must be mentioned that Mercodia approached our laboratory after reading the journal article on the comparisons between the ALPCO diagnostics and R&D systems kits (Sattar et al, 2008). Mercodia were keen to validate their new total adiponectin ELISA. We agreed to assist and in doing so were given permission to publish the results in this thesis.

Chapter V - High Molecular Weight Adiponectin and Incident Coronary Heart Disease: Findings from a prospective Case Control Study Nested within the British Women's Heart and Health Study.

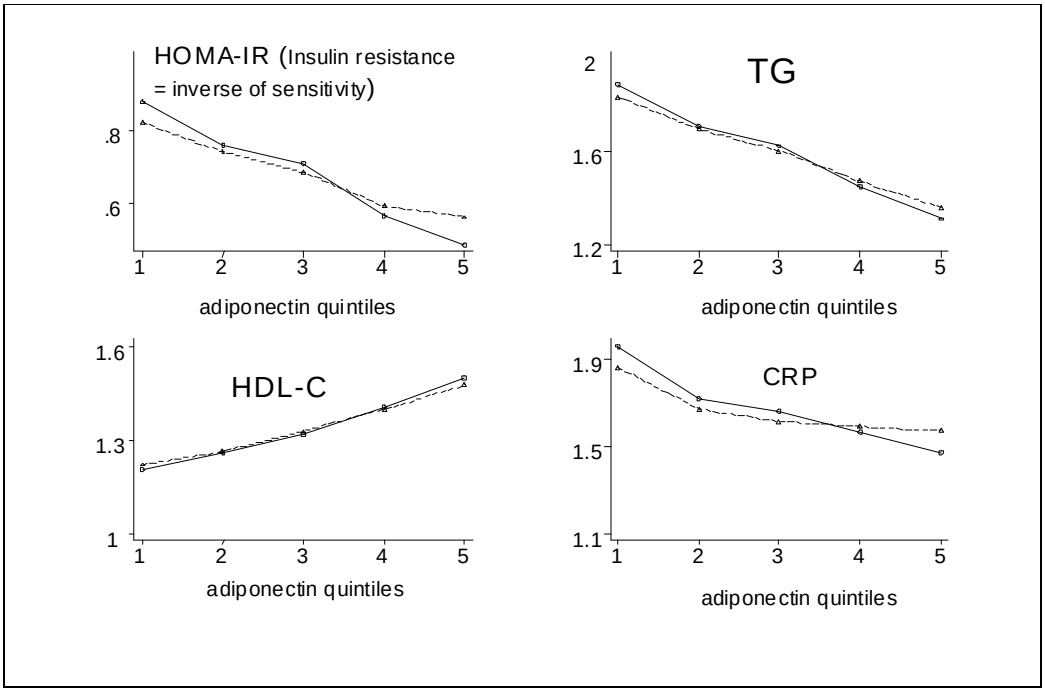
5.1 Introduction

In recent years, literature on the relationship of circulating levels of adiponectin to incident vascular events has attracted a lot of attention. The adipocyte derived hormone, adiponectin, has apparent anti-inflammatory (Gervois et al, 2007) and insulin sensitising properties (Kadowaki et al, 2006) therefore attracting considerable interest from research groups involved with CVD risk markers. Adiponectin has been shown to display a negative relationship with BMI, CRP and triglycerides, as well as a positive strong relationship with HDL-cholesterol (Figure 5.1) (Wannamethee et al, 2007). Whilst there is consistent evidence that high levels of adiponectin are associated with lower risk for incident diabetes (Lindsay et al, 2002; Wannamethee et al, 2007), it remains unclear whether high adiponectin levels are associated with lower risk for CHD events. One of the first studies to publish results on the relationship of adiponectin with CHD noted that high levels of total adiponectin were independently associated with lower risk for incident myocardial events (Vozarova et al, 2002). However since then other prospective studies have not been consistent. Our group showed no significant association of total adiponectin with incident CHD events in the British Regional Heart

study (BRHS), and in a subsequent meta-analysis of 7 prospective studies involving a total of 1318 CHD cases; including results from the British Women's Heart and Health Study (BWHHS) (Figure 5.2) (Sattar et al, 2006). Therefore, at present it is still unclear whether circulating levels of adiponectin are related to risk for incident vascular events.

Adiponectin is known to circulate in a number of different multimeric forms within our body, including a low, middle and high-molecular weight species. From recent data, it is thought that the latter HMW adiponectin species is the biologically active form of the molecule. HMW adiponectin has been shown to be better associated with insulin sensitivity than total adiponectin (Hara et al, 2006), be more strongly associated with incidence for diabetes (Nakashima et al, 2006), and it is also thought that the HMW moiety may be key to the vascular protective properties of the molecule, because it has been shown to be strongly linked to protection against endothelial cell apoptosis (Kobayashi et al, 2004). From this evidence, it is possible that the HMW form rather than the total adiponectin is more relevant to the risk for incident CHD events. Studies of this nature are still lacking. Until recently, the measurement of HMW adiponectin on a given sample was costly, complex and labour intensive. However, although still expensive, the introduction of an ELISA method for determination of separate adiponectin fractions has made it easier to measure HMW adiponectin on larger sample sets. Due to funding constraints we were not able to measure HMW adiponectin on all of the BWHHS samples and so we carried out a nested case control study taking baseline samples from women with subsequent CVD events and randomly selected control women of a similar age who did not suffer a CVD event.

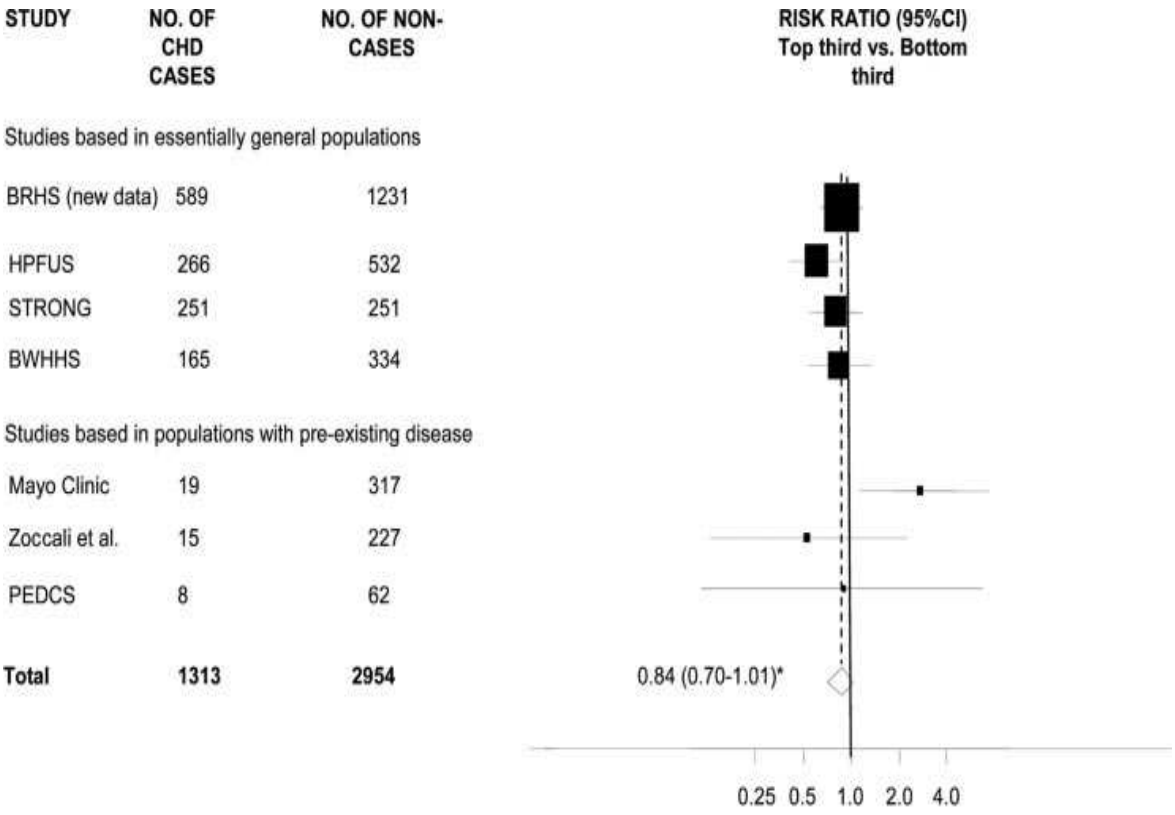
Figure 5.1: Graphs Showing the Relationship of Adiponectin with Various Parameters in Humans.



Abbreviations for figure 5.1: CRP, C reactive protein; HDL-C, High Density Lipoprotein Cholesterol; HOMA-IR, Homeostasis Model Assessment; TG, Triglyceride.

Figure 5.2: Figure Showing the 7 Prospective Studies of Circulating Concentrations of Adiponectin and CHD Risk.

We can see from the graph below the overall total meta-analysis shows no association between adiponectin levels and risk for CHD, because the confidence interval crosses 1. Note that this figure shows CHD risk ratios for those individuals in the top third of baseline adiponectin levels versus those in the bottom third. Pischon's study (HPFUS) (the first to report on the associations of adiponectin with CHD) shows a significant association between increased levels of adiponectin and myocardial events. As we can deduce from the data on this figure, studies since then have not been consistent with these findings. In particular, note the BRHS, the largest published study carried out on this relationship to date, demonstrates a non-significant association.



5.2 Principle

Our aim was to test whether HMW adiponectin was better associated with risk for incident vascular events than the previously reported total adiponectin from the original BWHHS (Lawlor et al, 2005).

5.3 Study Subjects and Methods

Blood collection, storage and analysis

The BWHHS is a prospective cohort study of 4,286 women randomly selected from 23 British towns between 1999 and 2001 and who were aged 60-79 years at baseline (Lawlor et al, 2002). The prospective case control study and end-point ascertainment nested within this cohort and used herein has been previously described (Lawlor et al, 2005). Briefly, women with CHD at baseline were excluded, and incident cases of CHD were identified through medical records and death registration up to January 2004. An incident case was defined as either of (i) death with an underlying or contributing cause of CHD (ICD10 codes I20-I25, I51.6); (ii) a myocardial infarction (WHO criteria), first diagnosis of angina or coronary artery by-pass or angioplasty. There were 167 incident cases of CHD (101 non-fatal and 66 fatal). Two controls were randomly selected, within 5-year age groups of the cases, from women without CHD at the baseline assessment. The BWHHS has Local- and Multi-Centre Research Ethics Committee (LREC and MREC) approvals and all participants provided written informed consent for their medical records to be reviewed.

Baseline blood samples were taken after a minimum six-hour fast. HMW adiponectin was measured by an ELISA on EDTA plasma samples (ALPCO

Diagnostics) (General methods section 2.4.2). This ELISA has been validated against the "gold standard" conventional fractionation, with excellent reported correlations (ρ = 0.94 to 0.97) between methods (http://www.alpco.com/pdfs/47/Human%20Multimeric%20Adiponectin%20Details.pdf). The intra-assay CV was 6%. Since we had previously measured total adiponectin in the same cohort by a different ELISA (R&D systems, UK) (General methods section 2.4.1.1), we assayed **total** adiponectin by the ALPCO method in a subset of 80 samples and compared this measurement to the results obtained two years previously using the R&D ELISA method: a correlation of r = 0.89 between methods was noted despite blinded measurements, conducted several months apart with different dilutions. These results concur with our findings that the R&D and ALPCO kits are well correlated for total adiponectin measurement in serum samples (chapter 4.3). Repeated sample freeze thaw did not influence HMW adiponectin values (chapter 3.2).

Methods for insulin, glucose, lipid levels and C-reactive protein and homeostasis model assessment of insulin resistance (HOMA-IR) have been detailed previously (Lawlor et al, 2002). All blood samples were taken between 08.00 and 18.00 with the time of sampling (to nearest minute) recorded. Levels of total adiponectin and HMW adiponectin did not vary by time of day of sampling (data not shown).

Statistical analysis

Associations of continuous covariables with HMW adiponectin and total adiponectin were assessed using Spearman's rank correlation coefficients.
Correlation coefficients are presented for the entire sample (cases and controls combined) as there was no evidence of these associations differing by case-control status. Differences between cases and controls

were assessed using an unpaired t-test for continuous variables and chi-squared test for categorical variables. Multiple logistic regression was used to assess the association of HMW adiponectin with CHD with adjustment for covariables. For positively skewed variables (HMW adiponectin, total adiponectin, CRP, glucose, insulin, HOMA-IR scores and triglycerides), geometric means and their 95% confidence intervals were used. Logged values were used for regression models. HMW adiponectin and total adiponectin were entered as continuous variable (natural logs) in these regression models. Regression coefficients for logged exposure variables are difficult to interpret and for this reason the effect estimates were expressed as the risk ratio of CHD for a doubling of HMW adiponectin or total adiponectin. Non-linear associations of quarters of the HMW adiponectin distribution were explored first as a series of three indicator variables and then as a continuous score and computing a likelihood ratio test comparing these two nested models. We repeated our analyses using conditional logistic regression and found the same results. All analyses were conducted in Stata version 9.0 (StataCorp., Texas).

5.4 Results

Of the randomly selected samples, one control did not have a baseline sample and therefore data was available for 167 cases and 333 controls. Table 5.1 shows the correlation of HMW with total adiponectin, giving an r = 0.75. Also from table 5.1 we can see both HMW adiponectin and total adiponectin were inversely associated with waist to hip ratio, fasting insulin, glucose, HOMA-IR, CRP and triglyceride levels and positively associated with HDL-c levels.

The 95% confidence interval levels of HMW adiponectin were not different between cases 4.07 (3.64, 4.55) μg/ml and controls, 4.17

(3.90, 4.47) µg/ml, where p = 0.7. As previously reported by Lawlor *et al* in 2005, cases had a higher fasting insulin, glucose, HOMA-IR, LDL-c and lower HDL-c. Triglyceride levels and systolic and diastolic blood pressure were similar. However hypertension and smoking were greater and use of HRT less at baseline in cases compared with controls.

In a multivariable analysis we found no association between HMW adiponectin and incident CHD (Table 5.2). The age-adjusted relative risk ratio for CHD for a doubling of HMW adiponectin was 0.96 (0.78, 1.18) (Table 5.2) and adjustment for any confounders did not alter this (model 2 and 3, Table 5.2).

Figure 5.3 shows the effect of HMW adiponectin by quarters of its distribution and from our examination we found no evidence of any linear or non-linear associations.

There was also no association of the ratio of HMW adiponectin:total adiponectin with CHD risk: age-adjusted relative risk per doubling of the ratio 1.10 (0.80, 1.50).

Table 5.1: Correlation of High Molecular Weight Adiponectin and Total Adiponectin with Measures of Adiposity, Insulin, Glucose and Lipid Levels in British Women aged 60-79 Years.

	Spearman rank correlations			
	HMW adiponectin	**p**	**total adiponectin**	**p**
HMW	-	-	0.75	< 0.001
BMI	-0.12	0.008	-0.20	< 0.001
WHR	-0.26	< 0.001	-0.32	< 0.001
Insulin	-0.35	< 0.001	-0.41	< 0.001
Glucose	-0.11	0.01	-0.20	< 0.001
HOMA-IR	-0.30	< 0.001	-0.38	< 0.001
CRP	-0.17	0.002	-0.20	< 0.001
HDL-C	0.37	< 0.001	0.44	< 0.001
Triglyceride	-0.34	< 0.001	-0.42	< 0.001

Abbreviations for Table 5.1: HMW, High Molecular Weight; BMI, Body Mass Index; WHR, Waist to hip Ratio; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-Reactive Protein, HDL-C, High Density Lipoprotein C.

Table 5.2: Multivariable Associations of High Molecular Weight Adiponectin with Incident Cases of Coronary Heart Disease (N=167 cases and 333 controls).

	Relative risk ratio (95% confidence interval) for a doubling of HMW adiponectin	**p linear trend**	**p non linear association**
Model 1: age adjusted[a]	0.96 (0.78-1.18)	0.71	0.96
Model 2: confounder adjusted[b]	0.98 (0.79-1.21)	0.85	0.79
Model 3: adjusted for confounders and potential mediators[c]	1.01 (0.81-1.24)	0.89	0.88

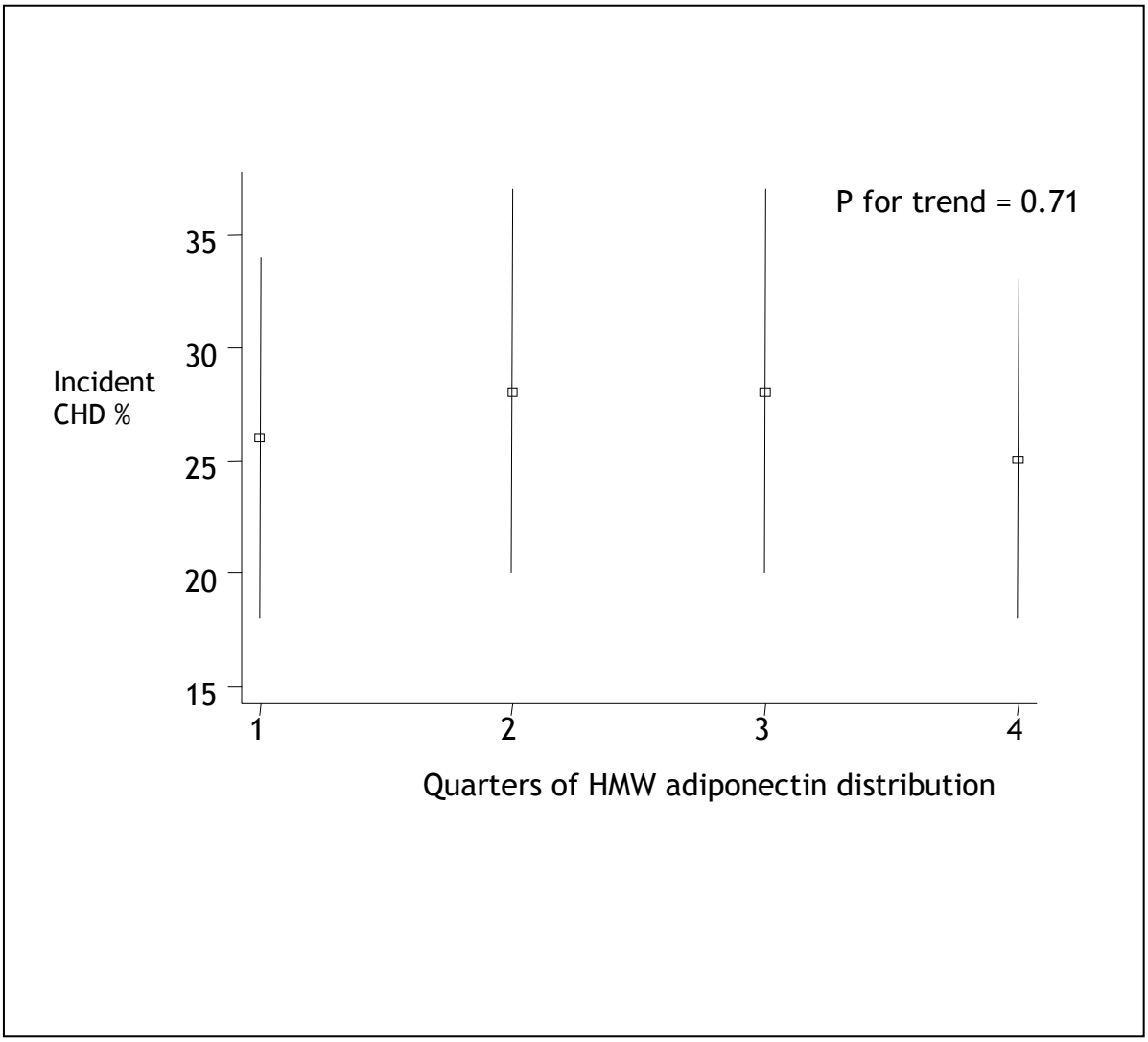
[a] **Model 1:** Adjusted for age (continuous in years)

[b] **Model 2:** as model 1 plus adult social class (4-level categorical variable entered as a score-1 parameter) smoking (3-level categorical variable entered as a score-1 parameter) and physical activity (3-level categorical variable entered as a score-1 parameter)

[c] **Model 3**: as model 2 plus, waist:hip ratio systolic blood pressure, fasting levels of high density lipoprotein, triglyceride (logged), glucose (logged), insulin (logged) and CRP (logged) (all continuous variable)

Figure 5.3: Proportion (%) of Women with Incidence Coronary Heart Disease (cases) by Quarters of the Distribution of High Molecular Weight Adiponectin.

The first quartile incorporates the bottom quarter of adiponectin values (i.e. those individuals having the lowest circulating adiponectin levels). The fourth quartile incorporates those women with the highest circulating levels of adiponectin. If there was a significant link between protection against CHD and levels of adiponectin, we would expect to see a linear relationship with those in the first quartile having a significantly higher incidence of CHD % than those in the fourth quartile. There is no evidence of any linear relationship here.



5.5 Discussion

A journal article on these findings has been published in the Journal of Clinical Endocrinology & Metabolism (JCEM) (Sattar et al, 2008) and includes the following contributing authors: Sattar N, Watt P, Cherry L, Ebrahim S, Davey Smith G, Lawlor DA (included in this thesis in appendix 4).

Our findings go against a clear link between HMW adiponectin and risk of incident vascular events. In line with our findings, Halperin et al reported no link between %HMW or total adiponectin and a vascular risk surrogate (endothelium-dependent vasodilation) in offspring of diabetic parents (Halperin et al, 2005). More interestingly, recent evidence suggests that higher adiponectin levels may actually predict higher risk for CVD mortality in the general population (Laughlin et al, 2007). HMW adiponectin predicts higher risk of mortality in patients with heart disease (Tsutsamoto et al, 2007), findings consistent with the observation by McEntegart *et al* in which they also reported a positive correlation between circulating adiponectin levels and BNP (McEntegart et al, 2007). In recent published literature (Kizer et al, 2008), it was demonstrated that higher levels of adiponectin predict increased risk of first ever CHD in older adults (inclusive of men and women). This latter study includes a large number of incident vascular events with good attention to potential confounders. These data suggest adiponectin and indeed its fractions have a complex relationship with vascular disease.

An editorial by Sattar and Nelson (Sattar et al, 2008) suggests reverse causality for the link between higher levels of adiponectin and the higher risk of CVD mortality. The editorial puts forward the notion that silent or clinically apparent vascular disease could lead to compensatory rises in adiponectin. Indeed adiponectin is thought to dampen the early phases of macrophage inflammatory responses, acting to inhibit the growth of myelomonocytic progenitor cells and decrease the ability of mature macrophages to respond to activation (Yokota et al, 2000; Gervois et al, 2007). This inhibited functioning of macrophages results in a reduced uptake of oxidised LDL, and this in turn inhibits foam cell formation. This is vital in fighting the onset of

atherosclerosis. Sattar and Nelson also mention the positive correlation adiponectin exhibits with BNP in patients with chronic heart failure, as well as those without, and alternately it could be that a rise in BNP may link silent ischemia or vascular disease to higher adiponectin levels. In line with this McEntegart and colleagues (McEntegart et al, 2007) recently proposed the idea that the high levels of adiponectin in those with heart failure or acute coronary syndrome may represent a salvage mechanism to improve insulin resistance and fatty acid metabolism, thereby attempting to counteract metabolic or vascular stress (Sattar et al, 2008). If this was indeed true, then an investigation into the link between adiponectin levels and mortality risks in those with chronic vascular disease should be a good indicator of the validity of these suggestions. One such study (Dekker et al, 2008) has touched on the subject and its findings point to a positive correlation between these two parameters.

Finally, although we expected the correlation between HMW adiponectin and HOMA-IR score to be greater than between total adiponectin and HOMA-IR given its apparent increased association with insulin sensitivity (Nakashima et al, 2006; Lawlor et al, 2005), this was not so in our study. Similar results have also been observed by other researchers (Retnakaran et al, 2007).

There may be possible limitations to our study that merit discussion. We understand that our study cannot be extrapolated to men and other ethnic groups and larger studies are needed to corroborate our null result, although the adjusted result of 1.01 (0.81, 1.24) for incident CHD events with a doubling of HMW adiponectin is close to unity (Sattar et al, 2008). Some may argue that using kits from two different suppliers for the measurement of total and HMW adiponectin may affect our results. However, although our method for determination of HMW adiponectin is fairly new, we believe it to be robust. It should be noted that Bluher *et al* questioned the robustness of this method in their report (Bluher et al, 2007). Their study included 60 women who spanned glycaemia thresholds and reported no association of total adiponectin by the ALPCO method with two other methods for total adiponectin, with their p value being reported just as non significant with no value assigned. They also noted

no association of either total or HMW adiponectin with fasting insulin levels (ρ = -0.09, and ρ = -0.04 respectively, both non-significant). Our study, by contrast, included 500 women without diabetes, and found the HMW fraction to correlate nicely with the total adiponectin (ρ = 0.75, $p<0.001$) using different methods for determination. Furthermore the total adiponectin measurement using the ALPCO kit correlated with the R&D ELISA on a subset of 80 samples (ρ = 0.89, $p<0.001$), even though the tests were done blinded and two years apart. In addition, the HMW fraction correlated with fasting insulin (-0.35, $p<0.001$) and with other metabolic parameters in the expected fashion (Table 5.1). Other groups have used gold standard techniques such as velocity sedimentation technique to report similar correlations between total and HMW adiponectin. For example Halperin *et al* (Halperin et al, 2005) reported a correlation of ρ =0.72 between %HMW and total adiponectin. Also, the ALPCO method has been validated against more conventional methods for adiponectin fractionation including immunoblotting and velocity sedimentation technique, and has been shown to perform well. Finally, others (von Eynatten et al, 2007; Hara et al, 2006) have used larger sized cross-sectional studies than that published by Bluher and colleagues, to show good performance of kits and a correlation to insulin sensitivity and related parameters. Therefore our null findings are unlikely to be due to methodological issues, although clearly more work in this area is required.

In conclusion, despite evidence to suggest that the HMW form of adiponectin is the biologically active form of the molecule, our results go against a significant inverse association between HMW adiponectin and incident CHD events. The findings from this study do however further support the notion that circulating adiponectin has a stronger association with diabetes than with vascular disease. Uncertainty still lies with the role adiponectin plays in vascular disease and more studies are required to investigate further.

Chapter VI - Serial Changes in Adiponectin and BNP in ACS Patients: Paradoxical Associations with Each Other and with Prognosis

6.1 Introduction

As mentioned previously throughout this thesis, adiponectin has been shown to have the unusual property of correlating inversely with markers of adiposity. It has also been suggested to possess anti-inflammatory properties (Ouchi et al, 2007) and in line with this may protect cardiac tissue and increase inflammatory dampening thereby limiting the occurrence of atherogenesis (Ouchi et al, 2006).

Despite adiponectin's clear link to lower diabetes risk, its relationship to CHD is not clear. An initial study linked low levels of adiponectin to increased risk of CHD events in healthy men (Pischon et al, 2004), however a recent meta-analysis suggested a more modest association with CHD (Sattar et al, 2006). Further to this, studies in people with prevalent heart failure or acute coronary syndromes show that high plasma levels of adiponectin are associated with **greater** disease severity (Nakamura et al, 2006; McEntegart et al, 2007) and with higher risk of adverse outcome (Tsutamoto et al, 2007; Kistorp et al, 2005; George et al, 2006; Cavusoglu et al, 2006). These studies highlight the complexity of the relationships adiponectin displays with regard to health and disease within the body. Circulating adiponectin concentrations may represent both a protective or theoretically harmful signal depending on the context.

Brain natriuretic peptide (BNP), now known as B-type natriuretic peptide (also BNP) is a 32 amino acid polypeptide secreted by the ventricles of the heart in response to excessive stretching of heart muscle cells (cardiomyocytes). BNP was originally identified in porcine brain and thereby got its name, but in humans it is found mainly in cardiac ventricles. BNP and its inactive N-terminal metabolite NT-proBNP (released in 1:1 ratio during processing) are sensitive markers of cardiac overload and are markers of prognosis in those

with CHF (Clerico et al, 2004). Baseline NT-proBNP and BNP have positive correlations with adiponectin in those with prevalent CHF and CAD (Nakamura et al, 2006; McEntegart et al, 2007; Tsutamoto et al, 2007; Kistorp et al, 2005; George et al, 2006; Cavusoglu et al, 2006; Clerico et al, 2004; von Eynatten et al, 2006; Tanaka et al, 2008). In particular the recent Atherogene study showed that baseline adiponectin predicted risk of poor outcome in CHF independent of conventional risk factors, but not independent of BNP (Schnabel et al, 2008).

To further investigate the potential pathological link between BNP and adiponectin, we examined in 442 ACS patients the inter-relationship of circulating adiponectin and BNP both at baseline and at a 7 week follow up (338 patients) with adverse outcomes at 10 months to determine whether change in either parameter was a better predictor of adverse events than a single measurement. Changes in concentrations of adiponectin over time were also investigated to see if they correlated with changes in BNP over time.

6.2 Materials and Methods

6.2.1 Study population

442 Caucasian patients with the diagnosis of ACS (between August 2004 and November 2006) were consecutively recruited at Ninewells Hospital, Dundee. Patients were recruited if they presented within 72 hours after the onset of ischemic discomfort and included those diagnosed with (Cnop et al, 2003) ST elevation MI (STEMI); ST elevation >1mm in 2 limb leads or >2mm in leads V1-V6 or new left bundle branch block, (Lafontan et al, 2006) Non ST segment elevation MI (NSTEMI); no ST elevation on ECG despite elevated Troponin T >0.01 ug/mL, (Ouchi et al, 2007) Unstable angina; ischaemic chest pain lasting more than 30 minutes with no evidence of myocyte necrosis or ST elevation. Ethical approval was obtained from the Tayside Committee of Medical Research Ethics and all participating subjects gave written, informed consent. The research was carried out in accordance with the Declaration of Helsinki. During the ACS admission, the patients underwent the following clinical procedures:

(1) Clinical history and risk factor analysis.
(2) Evaluation of admission Killip class defined as follow:
• **Killip class I**: No clinical signs of heart failure.

• **Killip class II**: Rales or crackles in the lungs, an S_3 gallop, and elevated jugular venous pressure.
• **Killip class III:** Frank acute pulmonary oedema.
• **Killip class IV:** Cardiogenic shock or hypotension (measured as systolic BP lower than 90 mmHg), and evidence of peripheral vasoconstriction (oliguria, cyanosis or sweating).
(3) ECG: presence of absence of ST deviation (>1.0 mm).
(4) Bedside BNP assay.
(5) Laboratory tests: admission haemoglobin, eGFR using the modification of diet in renal disease equation and serum Troponin-T level.
(6) Bedside echocardiography: LV systolic dysfunction (LVSD) defined as LVEF <45% (Simpson's biplane method) and left ventricular hypertrophy (LVH) assessment.

6.2.2 Echocardiography

Transthoracic echocardiography was performed by one trained operator using an
Acuson (Sequia 512) imaging system with a 3V2C transducer. The scan was performed with the patient lying in the left lateral position at approximately 45%.

6.2.3 Left ventricular hypertrophy assessment

Patients were studied with two-dimensional guided M-mode echocardiography in standard views. All measurements were made according to the American Society of Echocardiography (ASE) recommendation at end diastole, taken as the onset of QRS complex. The leading edge to leading edge convention was used to measure interventricular septal thickness, left ventricular internal diameter and left ventricular posterior wall thickness. Measurements were made over at least 3 separate cardiac cycles and the average taken. Left ventricular mass was calculated according to the formula of Devereux et al.

0.80 (ASE left ventricular mass) + 0.6 (Devereux et al, 1986) and indexed to body surface area to give a left ventricular mass index (LVMI). Left ventricular hypertrophy was defined as LVMI greater than 95g/m^2 in females and greater than 115 g/m^2 in males in accordance to ASE guidelines (Lang et al, 2005).

6.2.4 Left ventricular systolic function assessment

Quantitative assessment of LV systolic function was made using the modified biplane Simpson's method to calculate a LVEF. Three measurements from successive cardiac cycles were made in the two chamber and four chamber views. LVSD was defined as an LVEF < 45%.

6.2.5 Blood sample collection and analysis

Samples were collected by venipucture into EDTA vacutainer tubes or serum clot accelerators. One EDTA vacutainer of blood sample was kept at room temperature and analysed for BNP within 4 hours of the draw time. Whole blood was analysed with the triage BNP assay (Biosite, USA) as reported previously (Morrison et al, 2002). The inter-assay CV was 8.8% at 71.3 pg/mL and 11.6% at 4088 pg/mL. The detection limit was 5 pg/mL. An electrochemilumninescent immunoassay approved for quantitative measurement of Troponin T was provided by Roche Diagnostics and run on Roche Modular E170 unit. At 0.06 ug/L a CV of 10% is achievable and the detection limit was 0.01 ug/L. Blood samples for adiponectin or other non-routine analyte measurement were spun at 2000g for 15 min and the serum or plasma layers were aliquoted within 4 hours, snap frozen, and stored at –80°C. Total plasma adiponectin was analysed using a commercially available kit (R&D systems, Oxon, UK). The inter-assay CV for the adiponectin assay was less than 8%. The same methods were used for samples at follow-up measurements.

6.2.6 Follow-up Measurements

7 weeks after baseline admission, an attempt was made to re-examine patients with a full ECG, trans-thoracic echocardiography to assess LVEF, and routine blood tests including repeat bedside BNP and freezing of plasma

samples (average time of follow-up 52 ± 17 days). Of the 442 baseline patients (of whom 90 experienced adverse outcomes (detailed below) by 10 months), 433 were still alive, and 338 consented to continuing participation in the study at 7 weeks (of whom 51 experienced adverse outcome by 10 months). In relating BNP and adiponectin levels to risk of outcome we used all 442 baseline patients for whom measurements were obtained, and the 338 patient samples available to us from the 7 week measurement

6.2.7 End points

The end point of death from any cause, readmission with ACS or admission with congestive heart failure (CHF) was evaluated at 10 months (265.79 ± 80.31 days). Information on end points was collected from telephone interviews with patients or patient relatives, hospital database and patients case notes. The definition of readmission with ACS is as described above. Congestive heart failure was defined as hospitalization for a clinical syndrome involving at least 2 of the following: paroxysmal nocturnal dyspnoea, orthopnoea, elevated jugular venous pressure, pulmonary crackles, third heart sound and cardiomegaly or pulmonary oedema on chest X-Ray. These clinical signs and symptoms must have represented a clear change from the normal clinical status, requiring intravenous diuretics, inotropic support or vasodilator therapy.

Statistical analysis

Continuous variables are summarized as median and 25th and 75th percentile. For discrete variables, absolute and relative frequencies per category are given. Variables were logarithmically transformed to obtain normal distributions, with tertiles of adiponectin generated. Inter-group differences were assessed by chi squared test or analysis of variance. Spearman correlation coefficients are reported. The association of adiponectin with adverse outcome was examined in different binary logistic regression models, first a univariate model, then in a model adjusting for classical risk factors, a third model additionally adjusted for baseline BNP and a fourth model for 7 week BNP. Goodness of fit of the models was tested by Hosmer-Lemeshow. Statistical significance was determined at $p < 0.05$.

6.3 Results

6.3.1 Baseline characteristics

In the complete patient group the median BNP level at baseline was 154 pg/ml (inter-quartile range [IQR] 53-336), and the median baseline adiponectin level was 6.76mg/ml (IQR 4.16- 10.82). With continuous analyses, the baseline correlation between BNP and adiponectin was $\rho = 0.32$, ($p<0.001$).

Table 6.1 illustrates baseline characteristics of the patients according to tertiles of adiponectin concentration. As expected, age increased across tertiles of adiponectin (p for trend <0.001). Some other major cardiovascular risk factors showed inverse associations with adiponectin including proportion of male gender ($p<0.001$), BMI ($p<0.001$), and total cholesterol ($p=0.07$) while HDL cholesterol increased across tertiles ($p<0.001$). Blood pressure, presence of hypertension, diabetes, or smoking habit showed no significant association with adiponectin levels. Haemoglobin levels and kidney function (eGFR) decreased across adiponectin tertiles ($p<0.001$). Among clinical measures, left ventricle ejection fraction ($p<0.001$) decreased, and sub-optimal prognosis according to Killip class increased ($p=0.004$) with increasing adiponectin.

6.3.2 Changes in adiponectin and BNP from baseline to 7 weeks

The median BNP level at 7 weeks was lower compared to baseline at 94pg/ml (IQR 36-198) and the median adiponectin level was 5.49mg/ml (IQR 3.48-8.99). With continuous analyses the 7 week correlation between BNP and adiponectin was $\rho = 0.33$, ($p<0.001$). Overall change in adiponectin and BNP levels between baseline and 7 week follow up for the whole patient group are shown in Fig 6.1 a and b respectively. As can be seen from these figures both the change in adiponectin and BNP were near-normally distributed, with the former being slightly left-skewed and the latter right-skewed (skewedness statistic -1.22 and 1.80 respectively). For both biomarkers there was a spread of patients with either increases or decreases in either marker over the 7

week interval. Figure 6.2 (with both markers log-transformed to normality) demonstrates a significant correlation between changes in BNP versus change in adiponectin. Indeed the correlation (ρ =0.39, p<0.001) was at least as strong as the baseline and 7 week correlations between parameters noted above.

6.3.3 Associations of baseline adiponectin and BNP with adverse outcome

In unadjusted models, baseline adiponectin showed a borderline significant association with outcome: OR 2.06 (95% CI 0.92-4.63) p=0.08 (Table 6.2a). Adjusting for classical risk factors (including clinical parameters) attenuated the association (OR 1.63 [0.54 – 4.93]). Subsequent adjustments for BNP levels attenuated the association of baseline adiponectin with risk to near unity. As seen in Table 6.2b however, BNP remained significantly associated with poor outcome in every adjustment model, even after adjusting for baseline adiponectin (OR 3.26 [1.54 – 6.91]).

6.3.4 Associations of 7 week adiponectin and BNP with adverse outcome

Among those who survived the first 7 weeks following ACS and who consented to further study, the association between 7 week adiponectin and risk of poor outcome was not considerably different to the association seen at baseline, and none was significant, perhaps due to reduced power with fewer observations (Table 6.2a).In contrast, the association of 7 week BNP with risk of poor outcome was substantially stronger, and was independent of classical risk factors, and independent of 7 week adiponectin (OR 6.84 [2.54 – 18.45]) (Table 6.2b).

6.3.5 Associations of change in adiponectin and BNP with adverse outcome

Change in adiponectin (Table 6.2a), appeared somewhat more strongly linked to risk for adverse outcome than either baseline or 7 week adiponectin. For change in adiponectin the unadjusted OR was 5.42 (2.78 – 10.55), and attenuated to OR 3.99 (1.79 – 8.92) with adjustment for classical markers, and to OR 3.17 (1.29 – 7.78) with additional adjustment for change in BNP. Change in BNP was not itself independently associated with adverse outcome in the latter model, suggesting change in adiponectin was the stronger marker. Since change in adiponectin and 7 week BNP appeared to have the strongest independent associations with outcome, we included both markers in same multivariable model to test whether 7 week BNP could explain the association of change in adiponectin with prognosis. After doing this, change in adiponectin was no longer associated with risk for adverse outcome (OR 1.13 [0.27 – 4.92]), but 7 week BNP measurement remained significantly associated (OR 5.90 [1.04 – 32.94]).

Table 6.1: Association between admission adiponectin levels (divided into thirds) and baseline characteristics of ACS patients (n=442 with adiponectin measurement)

Characteristic		Bottom third 0.48-4.85µg/mL (n=147)	Middle third 4.86-8.94 85µg/mL (n=148)	Top third 8.95-35.40 85µg/mL (n=147)	p-value*
Age		60 (12)	66 (11)	69 (11)	<0.001
Sex Male/Female n (%)		125/22 (85/15)	98/50 (66/44)	77/70 (52/48)	<0.001
Systolic blood pressure (mmHg)		130 (115-150)	131 (118 – 150)	128 (110 – 147)	0.23
Diastolic blood pressure (mmHg)		70 (63-81)	72 (65 – 82)	70 (60 – 80)	0.18
Total cholesterol (mmol/L)		5.12 (4.19-6.08)	4.81 (3.94 –5.95)	4.52 (3.85-5.62)	0.07
HDL cholesterol (mmol/L)		1.15 (0.99-1.33)	1.32 (1.11-1.47)	1.50 (1.2 –1.82)	<0.001
BMI (kg/m^2)		28.0(26.0-31.0)	26.0 (24. – 29.0)	25.0 (22.0-29.0)	<0.001
Smoking n (%)	Never	46 (31)	57 (38)	52 (35)	
	Past	41 (28)	44 (30)	52 (35)	
	Present	60 (41)	47 (32)	43 (30)	0.21
Haemoglobin (g/dl)		15.0 (14.1 -15.8)	14.1 (12.7-14.9)	13.3 (12.1- 14.4)	<0.001
eGFR (mL/min/1.73m^2)		70 (59-78)	66 (52- 75)	61 (51- 73)	<0.001
Troponin T (ng/mL)		0.27 (0.01-1.86)	0.36 (0.06-1.47)	0.31 (0.07-1.43)	0.98
Non ST elevation ACS (%)		69	75	75	0.353
Type 2 Diabetes n (%)		21 (14)	23 (16)	16 (11)	0.48
History of hypertension(%)		61 (41)	71 (48)	74 (50)	0.29
ST deviation on ECG n (%)		73 (50)	68 (46)	71 (48)	0.81
LBBB on ECG n (%)		4 (2.7)	5 (3.4)	7 (4.8)	0.63
LV ejection fraction (%)		57 (49-63)	55 (45 – 62)	52 (39 – 61)	0.001
LV Hypertrophy (%)		84 (66)	81 (71)	74 (67)	0.680
Killip class 2,3 or 4 n (%)		8 (5)	13 (9)	25 (17)	0.004
BNP (pg/mL)		**81 (39-216)**	**152 (44-343)**	**239 (114-489)**	**<0.001**

Abbreviations for table 6.1: ACS, Acute coronary syndrome; eGFR, estimated glomerular filtration rate; LBBB, left bundle branch block; LV, left ventricular.
Data presented are the number (%) of patients or median and 25th/75th IQR for continuous variables. p-values represent trends across adiponectin third

Table 6.2a Association (Odds ratio) between baseline adiponectin, 7 week adiponectin, and change in adiponectin and collective adverse outcomes of mortality, heart failure or acute coronary syndrome (n=51) within 10 months of index acute coronary syndrome events.

	Adiponectin Measurement		
Adjustment model	**Baseline adiponectin**	**7 week adiponectin**	**Change in adiponectin**
Unadjusted	2.06 (0.92-4.63)	2.19 (0.79 – 6.08)	5.42 (2.78 – 10.55)
Classical risk factors	1.63 (0.54 – 4.93)	1.31 (0.33 – 5.27)	3.99 (1.79 – 8.92)
Classical risk factors + baseline BNP	1.18 (0.38 – 3.64)	-	-
Classical risk factors + 7 week BNP	-	0.74 (0.17 – 3.28)	-
Classical risk factors and change in BNP	-	-	3.17 (1.29 – 7.78)

ORs for adiponectin and BNP are for 1 log unit increase in the patient population.

Classical risk factors = Age, sex, BMI, pre-existing hypertension, pre-existing diabetes mellitus, Killip class 2,3 or 4, ST deviation, left bundle branch block, log troponin on admission, CKD stages 3, 4 or 5, left ventricular systolic dysfunction, smoking status, haemoglobin on admission, total and HDL cholesterol concentrations.

Table 6.2b Association (Odds ratio) between baseline BNP, 7 week BNP, and change in BNP and collective adverse outcomes of mortality, heart failure or acute coronary syndrome (n=51) within 10 months of index acute coronary syndrome events.

	BNP Measurement		
Adjustment model	**Baseline BNP**	**7 week BNP**	**Change in BNP**
Unadjusted	4.29 (2.59 – 7.11)	6.73 (3.33 – 13.60)	2.33 (1.13 – 4.82)
Classical risk factors	2.83 (1.35 – 5.92)	6.54 (2.49 – 17.21)	3.07 (1.25 – 7.53)
Classical risk factors + baseline adiponectin	3.26 (1.54 – 6.91)	-	-
Classical risk factors + 7 week adiponectin	-	6.84 (2.54 – 18.45)	-

ORs for adiponectin and BNP are for 1 log unit increase in the patient population.

Classical risk factors = Age, sex, BMI, pre-existing hypertension, pre-existing diabetes mellitus, Killip class 2,3 or 4, ST deviation, left bundle branch block, log troponin on admission, CKD stages 3, 4 or 5, left ventricular systolic dysfunction, smoking status haemoglobin on admission, total and HDL cholesterol concentrations.

Figure titles

Figure 6.1 (a&b): Dot-plots of the untransformed overall change in adiponectin and BNP between baseline observations and follow-up observations 7 weeks later in the patient group.

Figure 6.2: Correlation of change from baseline at 7 weeks follow-up for adiponectin and B-type natriuretic peptide. Delta values represent (log follow-up value – log baseline value) for both adiponectin and BNP.

Figure 6.1a

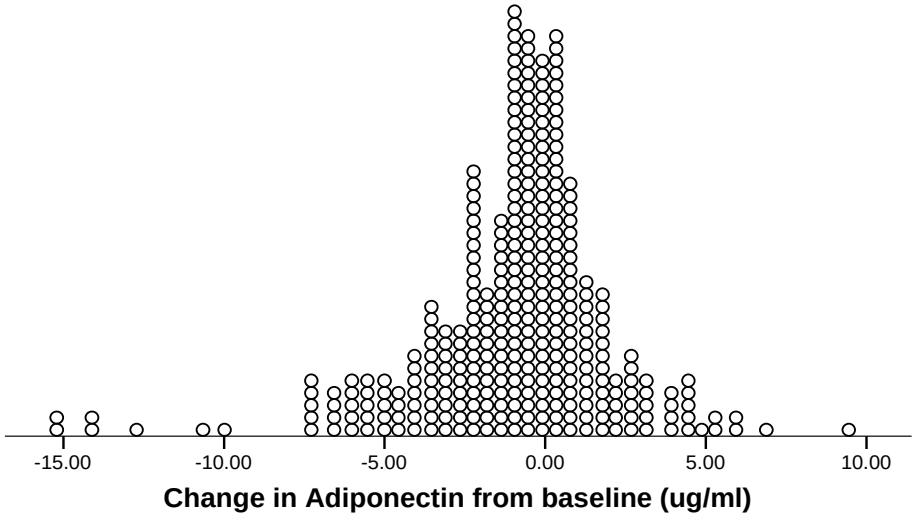


Figure 6.1b

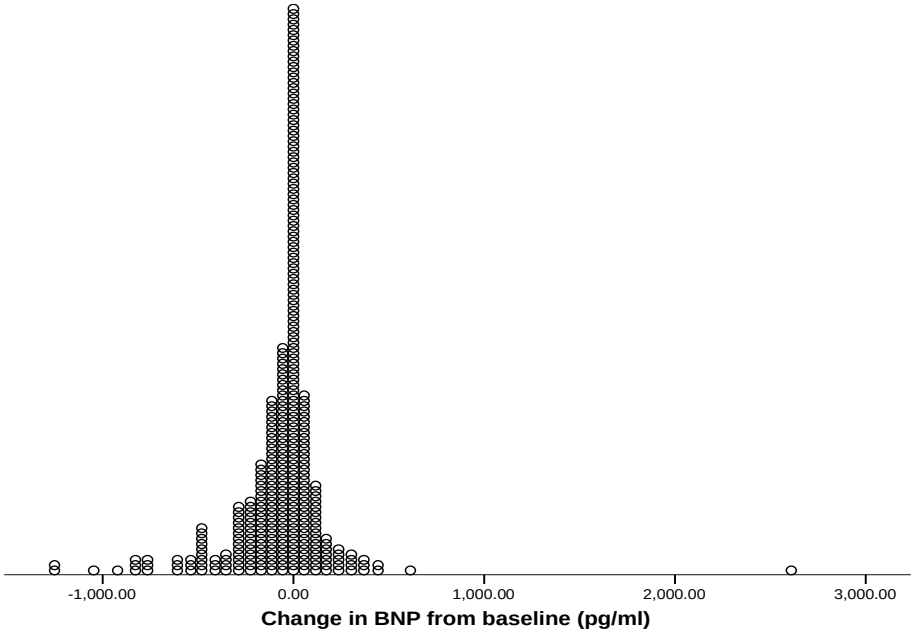
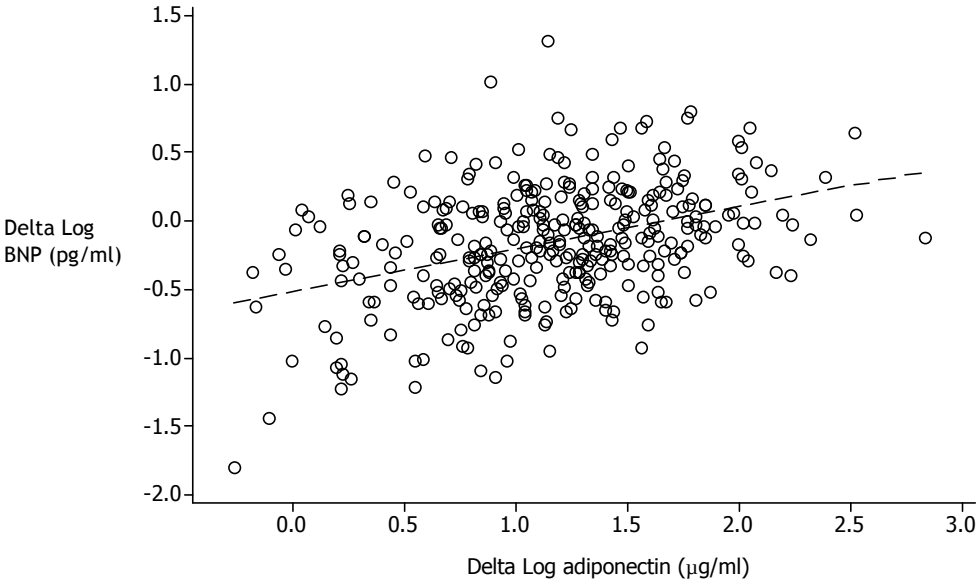


Figure 6.2



6.4 Discussion

A journal article has been published on these findings in Clinical Science London (Ang et al, 2009) and includes the following contributing authors: Ang DS, Welsh P, Watt P, Nelson SM, Struthers A, Sattar N (included in this thesis in appendix 4).

To our knowledge, this is the first study to date to examine the association of circulating adiponectin and BNP with risk of poor outcome in a group of ACS patients where serial measurements have been made. Importantly, an increase in adiponectin 7 weeks after admission was more strongly associated with risk of adverse outcome than a single measure at either time point – in other words, if adiponectin levels increased, adverse event risk was greater, whereas the absolute levels of adiponectin at either time point were less important. In contrast, 7 week BNP measurement was more strongly associated with risk, superior to both baseline and change in BNP measurements. In terms of independent risk prediction, the strongest marker of risk of adverse events was 7 week BNP, which was associated with risk independently of change in adiponectin. Finally, although the patterns of risk associations of these markers are different, these data allow us to show that following index admission for ACS, BNP and adiponectin associate with each other at baseline, at follow up, and that change in both markers also correlate (ρ =0.39, $p<0.001$). Our finding of a positive correlation between baseline circulating BNP and adiponectin, although apparently paradoxical on the basis of presumed functions of adiponectin, was an expected result based on previous findings in similar patient groups (Nakamura et al, 2006; McEntegart et al, 2007; Tsutamoto et al, 2007; Kistorp et al, 2005; George et al, 2006; Cavusoglu et al, 2006; von Eynatten et al, 2006; Tanaka et al, 2008; Schnabel et al, 2008). Our finding that 7 week BNP is more strongly related to adverse outcome than BNP at admission is in agreement with a recent study in 157 CHF patients, where discharge BNP was shown to be strongly associated with poor outcome (Cournot et al, 2008). We do not propose that our results definitively answer the question of whether circulating BNP and adiponectin are or are not clinically useful in prognosis of ACS. Rather, our results may give insight into the pathophysiology behind increased risk of ACS recurrence,

CHF, or death following an index ACS event. Our results are suggestive of an interesting and potentially important pathphysiological pathway which links an increase in adiponectin to adverse prognosis in a manner not independent of BNP. Although an observational study cannot imply a causal link, it is interesting to speculate on the potential relationship of adiponectin and BNP following ACS. We found that absolute levels of BNP at 7 weeks were the strongest predictor of prognosis. This makes sense, since levels of BNP following the settling of acute phase response and/or changes in clinical therapies after an event will better represent long-term cardiac /vascular status, and therefore subsequent risk. In contrast to BNP, increasing levels of adiponectin following index ACS admittance were potentially more strongly related to risk than baseline or 7 week adiponectin, and yet this relationship was partially dependant on 7 week BNP in multivariable models. This may be suggestive of some association between progressive disease severity and changes in adiponectin (as measured by follow-up BNP). The Atherogene investigators have very recently shown that the relationship of baseline adiponectin with risk of death or non-fatal MI in a group of coronary artery disease patients is confounded by associations with BNP (Schnabel et al, 2008). Our observations now extend this observation to show that the two parameters also change in parallel post ACS, such that the risk associations of adiponectin with risk are dependant on BNP. This provides evidence that there may be a direct or indirect link between high post ACS BNP and a rise in adiponectin in those at greatest risk of poor outcome. If there is a link between the two parameters, are there any clues to mechanisms? Natriuretic peptides may directly stimulate higher adiponectin levels since a novel lipolytic and potential lipid-mobilising effect of natriuretic peptides has been identified (Sengenes et al, 2000). These actions appear to be mediated by specific adipocyte membrane receptors, which operate via a cGMP-dependent pathway and they may indirectly stimulate adiponectin production (Sengenes et al, 2000). Furthermore, infusion of atrial natriuretic peptide (carperitide) in patients with heart failure leads to increased adiponectin plasma levels (Tanaka et al, 2008). Alternatively, TNFα (among other pro-inflammatory cytokines) has been suggested to inhibit adiponectin expression in tissue from healthy subjects (Bruun et al, 2003; Wang et al, 2006), but perhaps not in obese subjects (Degawa-Yamauchi et al, 2005). TNFα is known to be elevated

in conditions such as CHF and ACS (Mizia-Stec et al, 2003), and hence, speculatively, the inhibitory signal may also be lost in such patients. A recent attempt at explaining the physiology behind the reverse epidemiology of adiponectin in patients with ACS and CHF has been made, with the authors suggesting that elevated levels of adiponectin in these cases represent an attempt at counter-regulation of systemic inflammation (Rathmann et al, 2000). Although this is a possibility, the additional possible direct cardio-protective and metabolic roles of adiponectin in ACS and CHF should not be overlooked. Importantly, adiponectin may directly reduce oxidative ischemia-reperfusion injury (Shibata et al, 2005; Tao et al, 2007; Gonon et al, 2008), perhaps alluding to a mechanism whereby elevated BNP, in response to cardiac injury, induces an adiponectin-mediated cardio-protective response. Interestingly, this may also be true in silent ischaemia since others have suggested elevations in BNP may also occur in silent ischaemia (Rana et al, 2006; Struthers et al, 2007), an observation potentially explaining why it is a better marker than simple echo measures of cardiac dysfunction. Additionally, from a metabolic viewpoint, ACS and CHF are often considered to be insulin resistant states, although individuals who are insulin resistant without cardiovascular complications would be expected to have low levels of adiponectin. As we recently suggested (McEntegart, 2007), high levels of adiponectin in those with ACS and CHF may be a reflection of a salvage mechanism to improve insulin resistance and fatty acid oxidation, perhaps at a cost of cachexia in some cases. Regardless of mechanisms, greater cardiac disease severity may lead to a greater cardiological and metabolic salvage attempt and thus higher adiponectin levels. Alternatively, our speculation may be misguided and adiponectin may just be a passive marker of other physiological processes, its actions inhibited by peripheral “adiponectin resistance.” Limitations of the study require consideration. The group of 442 ACS patients went on to have 90 endpoint events, and follow-up measures are based on 51 events in 338 patients, which represents a relatively low power for the study and we cannot discount the possibility of type II errors. Nevertheless, our data on baseline associations of adiponectin with age and female gender (Balion et al, 2008) as well as with BNP (Nakamura et al, 2006; McEntegart et al, 2007; Tsutamoto et al, 2007; Kistorp et al, 2005; George et al, 2006; Cavusoglu et al, 2006; von Eynatten et al, 2006; Tanaka et al, 2008;

Schnabel et al, 2008), and the link to endpoints (Schnabel et al, 2008; Cournot et al, 2008; Vorovich et al, 2008), is consistent with the literature and suggest results are externally valid. Further larger studies with serial measures are required to confirm and expand on the potential mechanistic relationships of adiponectin, BNP, and cardiac stress. In this study we do not focus on the potential clinical utility of any measure of adiponectin or BNP in the ACS clinical setting, but on pathophysiological changes in ACS which may instruct further clinical studies.

Of additional interest, the use of genetics (e.g. Mendelian randomisation) may help tease out the whether adiponectin is protective, harmful, or passive for vascular risk for a variety of patient/cohort groups. In conclusion, we have shown for first time by using serial measurements that an increase in plasma adiponectin after ACS is strongly related to risk of adverse outcome, but that this relationship is not independent of BNP levels. Taken together with evidence of apparently paradoxical correlations of adiponectin and BNP at baseline, follow-up, and in overall change following ACS, our results allude to a potential direct or indirect relationship between adiponectin and BNP post-ACS that requires further investigation.

Chapter VII: Discussion

Adiponectin has become a popular molecule for investigation in the ongoing search for novel risk markers of diseases associated with the metabolic syndrome. It displays insulin sensitising properties as well as having apparent anti-inflammatory functions. Although it is predominantly released from fat cells, its levels are reduced in obese individuals, and the hypothesis is that mechanisms associated with insulin resistance, such as inflammation or altered adipocyte morphology may be the reason for this. Since the first description of the molecule in 1995, the precise role this adipokine plays in the onset of disease has been difficult to determine, making it such an exciting molecule for investigation.

Many prospective studies have been carried out to determine the role adiponectin plays in health and disease, but few have considered the laboratory aspects. This thesis has tested adiponectin's robustness in laboratories and it's associations with CVD using prospective studies. The results have shed new insights of potential relevance. The content of this thesis has highlighted the importance of considering pre-analytical (Results summarised in table 7.1) and practical variables in large epidemiological and clinical studies. I have shown that one measurement of adiponectin is sufficient for use in large epidemiological studies. The reproducibility in measurement of this molecule is good and the biological variation is minimal compared to some of the other inflammatory cytokines. From our studies, freeze/thaw cycles do not affect adiponectin concentrations; we recommend further investigation on this. Results from studies involving varied blood processing times and temperatures revealed no significant difference in samples left un-separated at both room temperature (23˚C-25˚C) and in the cold room (4˚C -7˚C) for up to 6 days. I have validated the use of citrated plasma samples on a commercial ELISA (R&D systems adiponectin assay) in our lab, and have also shown that 3 adiponectin ELISA kits developed by different suppliers correlate well with one another. This is important for the comparison of results between studies that have employed different commercial kits for adiponectin analysis, although some absolute differences suggest the same assay should be used throughout any individual study.

Table 7.1 Overview of pre-analytical variables for total and HMW adiponectin measurement.

	Sample Type		Accuracy		Sample Processing				Freeze-thaw		Variability
	Use in Serum?	Use in Plasma?	Intra Assay CV	Inter Assay CV	Absolute Values Stable?		Correlations Stable?		Absolute Values Stable?	Correlations Stable?	R Coefficient (95% CI)
					Fridge	Bench	Fridge	Bench			
									(Pilot study/ larger study)		
Total Adiponectin	+	+*	< 5%	< 7%	+	+	+	+	+/-	+/+	0.99 (0.98 – 1.00)
									(Only tested on pilot study)		
HMW Adiponectin	+	+	< 5%†	< 6%†	?	?	?	?	+	+	0.96 (0.78–1.18) ‡

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* Also validated in this thesis (chapter 4.2) for use with Citrate Plasma

† Data taken from ALPCO Diagnostics SOP for the quantitative, selective determination of high, middle and total molecular weight adiponectin in human serum or plasma

‡ Data taken from Sattar et al, 2008

With regards to the clinical studies, our published work as presented in detail herein has shown that:

I. HMW adiponectin is not predictive of CHD events.
II. Adiponectin change post ACS correlates with change in BNP and elevations in both parameters predict incident events, with BNP associations potentially mediating adiponectin's link to incident events.

This work therefore extends current knowledge on both laboratory aspects of adiponectin and its associations with vascular disease, although of course, I have not examined causal mechanisms but merely reported associations.

Further work

1. Ideally it would be useful to extend on our laboratory data by validating the ALPCO HMW adiponectin ELISA against the gold standard method (ultracentrifugation) on a large set of samples (Ideally n>100) to confirm our findings.

2. Further work is required to examine the relationship between HMW adiponectin and CHD. Although our study found no link between HMW adiponectin and risk of incident vascular events, a larger prospective study (therefore offering more statistical power) is needed to confirm our findings.

3. Although research into not mentioned in this thesis, it is still very important to note that underlying genes may be responsible for the relationships reported in several studies. Whether future studies using genetic variations in adiponectin concentrations help determine causal pathways, remains to be proven.

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Website last modified and checked: 4 August 2010

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APPENDICES

Appendix 1

Manufacturers and suppliers of reagents, hardware and software

1. Reagents and kits

All R & D kits

R & D Systems Europe Ltd
19 Barton Lane
Abingdon Science Park
Abingdon
OX14 3NB
United Kingdom
Tel: +44 (0) 1235 529449
Fax: +44 (0) 1235 533420
Email: info@RnDSystems.co.uk

All ALPCO Diagnostics kits

Diagenics Limited
South House 3
Bond Avenue
Bletchley
Milton Keynes
MK1 1SW
United Kingdom
Tel: 01908 376376
Fax: 01908 376375
Email:cs@alpco.com

Mercodia Kits

Head office in Uppsala, Sweden:
Sylveniusgatan 8A
SE-754 50 Uppsala, Sweden
E-mail: info@mercodia.com
Tel: +46 18 57 00 70
Fax: +46 18 57 00 80
Netherlands Contact: Mattias Gareskog
E-mail: mattias.gareskog@mercodia.se
Tel: +46 18 57 00 77

2. Equipment

Hamilton Diluter (Microlab 500 series)
Scientific Laboratory Supplies Ltd
Unit 17
Coatbridge Business Centre
204 Main Street
Coatbridge
Lanarkshire
ML5 3RB
United Kingdom
Tel: (01236) 431857
Fax: (01236) 431050
Email: info@scientific-labs.com

Multiskan Ascent Plate Reader and Ascent Software, Denley minimix Plateshaker and Gilson pipettes and Multichannel pipette.
Thermo Life Sciences
Unit 5
The Ringway Centre
Edison Road
Basingstoke
Hampshire
RG 21 6YH
Tel: (01256) 817282
Fax: (01256) 817292
Website: www.thermols.com

Blood Bottles (Vacutainers)
BD
The Danby Building
Edmund Halley Road, Oxford Science Park
Oxford
OX4 4DQ
United Kingdom
Tel: +44 1865 748844
Fax: +44 1865 717313
Email: bdukvacutainer@europe.bd.com
Website: www.bd.com/uk

Versa max Plate reader
Molecular Devices Ltd
Unit 135, Wharfedale Road
Winnersh
Wokingham
Berkshire
RG41 5RB
Tel: 0118 944 8000
Fax: 0118 9448001
Website: www.moleculardevices.com

Appendix 2

Calculation of Biological CV

Total CV = Total intra-person variation (12.9%)
Methodological CV = Intra-assay CV (4.07%)

$$\text{Biological CV} = \frac{\text{Total CV} - \text{Methodological CV}}{1 - \text{Total CV}}$$

$$= \frac{0.129 - 0.0407}{1 - 0.129}$$

$$= \frac{0.0883}{0.871}$$

$$= \mathbf{10.14\ \%}$$

Appendix 3
Kit Inserts

R&D Systems ELISA for total adiponectin determination

Mercodia ELISA for total adiponectin determination

ALPCO Diagnostics ELISA for total, HMW, MMW and LMW adiponectin determination

Quantikine®

Human Total Adiponectin/Acrp30 Immunoassay

Catalog Number DRP300
SRP300
PDRP300

For the quantitative determination of total human Adiponectin concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

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614 McKinley Place NE
Minneapolis, MN 55413
United States of America

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INTRODUCTION

Adiponectin, alternately named Adipocyte complement-related protein of 30 kDa (Acrp30), adipoQ, adipose most abundant gene transcript 1 (apM1), and gelatin-binding protein of 28 kDa (GBP28), is an adipocyte-specific, secreted protein with potential roles in glucose and lipid homeostasis. Circulating Adiponectin levels are high, accounting for approximately 0.01% of total plasma protein (1 - 4). Adiponectin contains a modular structure that includes an N-terminal collagen-like domain followed by a C-terminal globular domain with significant sequence and structural resemblance to the complement factor C1q (1, 5, 6). Although they share little sequence identity, similar three-dimensional structure and certain conserved amino acid residues suggest an evolutionary link between the C1q-like domain of Adiponectin and members of the TNF superfamily (7). Adiponectin assembles into different complexes including trimers (low molecular weight), hexamers (middle molecular weight), and higher order oligomeric structures (high molecular weight) that may affect biological activity (1, 7, 8). Adiponectin is induced during adipocyte differentiation and its secretion is stimulated by insulin (1, 9). Two receptors for Adiponectin, termed AdipoR1 and AdipoR2, have been cloned (10). Although functionally distinct from G-protein-coupled receptors, the genes encode predicted proteins containing 7 transmembrane domains. AdipoR1 is highly expressed in skeletal muscle, while AdipoR2 is primarily found in hepatic tissues.

Injection of Adiponectin into non-obese diabetic mice leads to an insulin-independent decrease in glucose levels (11). This is likely due to insulin-sensitizing effects involving Adiponectin regulation of triglyceride metabolism (11). A truncated form of Adiponectin (gAdiponectin) containing only the C-terminal globular domain has been identified in the blood, and recombinant gAdiponectin has been shown to regulate weight reduction as well as free fatty acid oxidation in mouse muscle and liver (2, 12). The full-length recombinant Adiponectin protein is apparently less potent at mediating these effects (2, 12). The mechanism underlying the role of Adiponectin in lipid oxidation may involve the regulation of expression or activity of proteins associated with triglyceride metabolism including CD36, acyl CoA oxidase, AMPK, and PPARγ (12 - 14).

Although Adiponectin-regulation of glucose and lipid metabolism in humans is less clear, similar mechanisms may also be in place (15). A negative correlation between obesity and circulating Adiponectin has been well established (6, 16, 17), and Adiponectin levels increase concomitantly with weight loss (18). Decreased Adiponectin levels are associated with insulin resistance and hyperinsulinemia, and patients with type-2 diabetes are reported to exhibit decreased circulating Adiponectin (19, 20). Thiazolidinediones, a class of insulin-sensitizing, anti-diabetic drugs, elevate Adiponectin in insulin-resistant patients (21). In addition, high Adiponectin levels are associated with a reduced risk of type-2 diabetes (22). Using magnetic resonance spectroscopy it has been demonstrated that intracellular lipid content in human muscle negatively correlates with Adiponectin levels, potentially due to Adiponectin-induced fatty acid oxidation (15).

Adiponectin may also play anti-atherogenic and anti-inflammatory roles. Adiponectin plasma levels are decreased in patients with coronary artery disease (20). Furthermore, neointimal thickening of damaged arteries is exacerbated in Adiponectin-deficient mice and is inhibited by exogenous Adiponectin (23). Adiponectin inhibits endothelial cell expression of adhesion molecules *in vitro*, suppressing the attachment of monocytes (24). In addition, Adiponectin negatively regulates myelomonocytic progenitor cell growth and TNF-α production in macrophages (25, 26).

The Quantikine Human Total Adiponectin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure total (low, middle, and high molecular weight) human Adiponectin in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human Adiponectin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Adiponectin showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human Total Adiponectin kit can be used to determine relative mass values for naturally occurring Adiponectin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the Adiponectin globular domain has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Adiponectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for the Adiponectin globular domain is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Adiponectin bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Description	Part #	Cat. # DRP300	Cat. # SRP300
Adiponectin Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against the Adiponectin globular domain.	892517	1 plate	6 plates
Adiponectin Conjugate - 21 mL/vial of mouse monoclonal antibody against the Adiponectin globular domain conjugated to horseradish peroxidase with preservatives.	892518	1 vial	6 vials
Adiponectin Standard - 500 ng/vial of recombinant human Adiponectin in a buffered protein base with preservatives; lyophilized.	892519	1 vial	6 vials
Assay Diluent RD1W - 11 mL/vial of a buffered protein base with preservatives.	895117	1 vial	6 vials
Calibrator Diluent RD5-5 - 21 mL/vial of a buffered protein base with preservatives.	895485	1 vial	6 vials
Calibrator Diluent RD6-39 - 21 mL/vial of a buffered protein base with preservatives.	895824	3 vials	18 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
Plate Covers - Adhesive strips.	—	4 strips	24 strips

DRP300 contains sufficient materials to run an ELISA on one 96 well plate.
SRP300 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDRP300). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="10">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="9">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1W</td></tr>
<tr><td>Calibrator Diluent RD5-5</td></tr>
<tr><td>Calibrator Diluent RD6-39</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for serial dilution.
- Human Total Adiponectin Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6-39 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 100-fold dilution. A suggested 100-fold dilution is 10 µL sample + 990 µL Calbrator Diluent RD6-39.

REAGENT PREPARATION

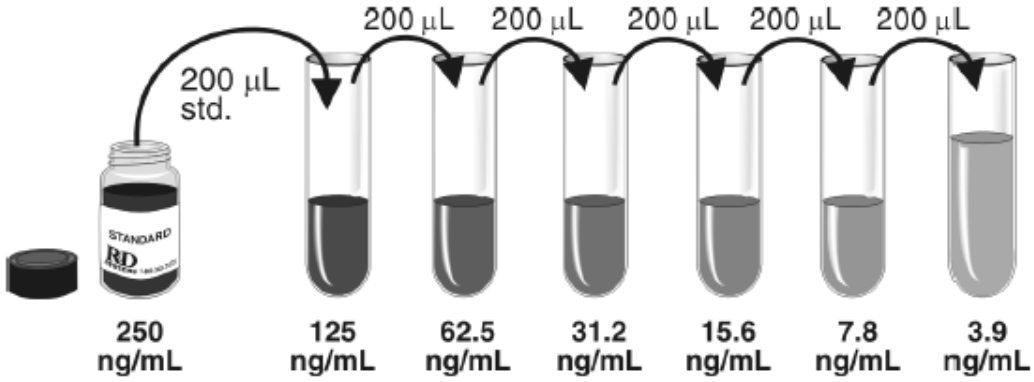
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Adiponectin Standard - Reconstitute the Adiponectin Standard with 2.0 mL of Calibrator Diluent RD5-5 *(for cell culture supernate samples)* or Calibrator Diluent RD6-39 (*for serum/plasma samples)*. This reconstitution produces a stock solution of 250 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-5 (*for cell culture supernate samples*) or Calibrator Diluent RD6-39 (*for serum/plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 250 ng/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 µL of Assay Diluent RD1W to each well.

4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of Adiponectin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well.
   For cell culture supernate samples: Incubate for **20 minutes** at room temperature.
   For serum/plasma samples: Incubate for **30 minutes** at room temperature.
   Protect from light.

9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Serum/plasma samples require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 100 µL Assay Diluent RD1W to each well.

3. Add 50 µL Standard, control or sample* to each well. Incubate 2 hours at RT.

4. Aspirate and wash 4 times.

5. Add 200 µL Conjugate to each well. Incubate 2 hours at RT.

6. Aspirate and wash 4 times.

7. Add 200 µL Substrate Solution to each well. **Protect from light.**

Serum/Plasma samples: Incubate 30 minutes at RT.

Cell Culture Supernate samples: Incubate 20 minutes at RT.

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 540 or 570 nm

*Serum/plasma samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

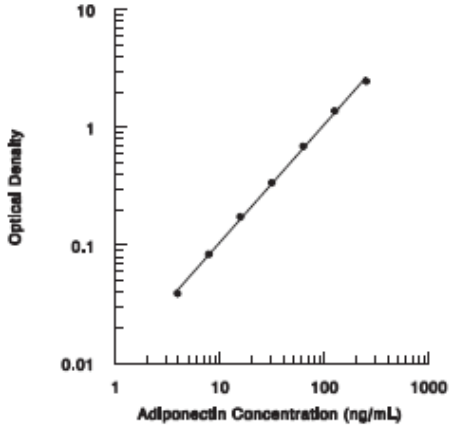
To determine the Adiponectin concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding Adiponectin concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

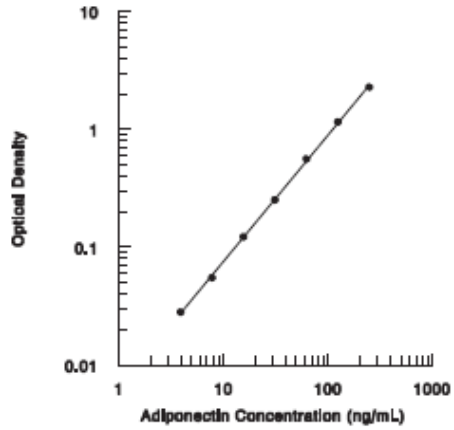
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5-5



ng/mL	O.D.	Average	Corrected
0	0.011 0.012	0.012	—
3.9	0.051 0.051	0.051	0.039
7.8	0.092 0.100	0.096	0.084
15.6	0.185 0.187	0.186	0.174
31.2	0.339 0.362	0.351	0.339
62.5	0.692 0.711	0.702	0.690
125	1.382 1.401	1.392	1.380
250	2.464 2.496	2.480	2.468

Calibrator Diluent RD6-39



ng/mL	O.D.	Average	Corrected
0	0.013 0.014	0.014	—
3.9	0.042 0.042	0.042	0.028
7.8	0.068 0.070	0.069	0.055
15.6	0.132 0.140	0.136	0.122
31.2	0.248 0.282	0.265	0.251
62.5	0.572 0.573	0.573	0.559
125	1.155 1.175	1.165	1.151
250	2.216 2.356	2.286	2.272

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	19.8	69.9	143	20.5	74.4	157
Standard deviation	0.50	2.40	6.76	1.40	4.30	10.8
CV (%)	2.5	3.4	4.7	6.8	5.8	6.9

Cell Culture Supernate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	12.5	45.3	91.5	12.7	47.1	100
Standard deviation	0.41	1.27	4.56	1.00	2.80	6.70
CV (%)	3.2	2.8	5.0	7.9	5.9	6.7

RECOVERY

The recovery of Adiponectin spiked to levels throughout the range of the assay was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	101	88 - 112

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Adiponectin were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)
1:2	Average % of Expected	104	99	99	95
	Range (%)	101 - 107	92 - 103	93 - 106	86 - 102
1:4	Average % of Expected	103	98	99	96
	Range (%)	101 - 106	91 - 101	92 - 109	89 - 104
1:8	Average % of Expected	103	102	104	97
	Range (%)	101 - 104	98 - 105	97 - 107	84 - 102
1:16	Average % of Expected	103	105	104	101
	Range (%)	100 - 104	96 - 115	103 - 105	85 - 107

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of Adiponectin ranged from 0.079 - 0.891 ng/mL. The mean MDD was 0.246 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Adiponectin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples drawn from apparently healthy volunteers were evaluated for the presence of Adiponectin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=60)	6641	865 - 21,424	3665
EDTA plasma (n=35)	5548	1198 - 19,973	3557
Heparin plasma (n=35)	6026	1359 - 20,691	3728

Cell Culture Supernates - Human peripheral blood leukocytes (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernate were removed and assayed for levels of natural total Adiponectin. No detectable levels were observed.

SPECIFICITY

This assay recognizes recombinant and natural (low, middle, and high molecular weight) human total Adiponectin. The factors listed below were prepared at 50 ng/mL in Calibrator Diluents RD5-5 and RD6-39 and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Adiponectin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
4-1BB
APRIL
BAFF/BLyS
CD27
CD30 Ligand
CD40 Ligand
Fas Ligand
GITR Ligand
LIGHT
LT-α1/β2
LT-α2/β1
OX40 Ligand
TNF-α
TNF-β
TRAIL
TRANCE
TWEAK
VEGI

Recombinant mouse:
Adiponectin
CD27 Ligand
CD30 Ligand
Fas Ligand
LT-α1/β2
LT-α2/β1
OX40 Ligand
TNF-α
TNF-α (truncated)
TRANCE

Recombinant porcine:
TNF-α

Recombinant rat:
TNF-α

Other proteins:
human Complement C1q

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Mercodia Adiponectin ELISA

Directions for Use

10-1193-01
REAGENTS FOR 96 DETERMINATIONS

For Research Use Only
Not for Use in Diagnostic Procedures

Manufactured by

Mercodia AB
Sylveniusgatan 8A
SE-754 50 Uppsala
Sweden

Mercodia

EXPLANATION OF SYMBOLS USED ON LABELS

	Reagents for 96 determinations
	Expiry date
	Store between 2-8°C
	Lot No.

INTENDED USE

Mercodia Adiponectin ELISA provides a method for the quantitative determination of human adiponectin in serum or plasma.

SUMMARY AND EXPLANATION OF THE TEST

Adiponectin is also called: Acrp30 (30 kDa adipocyte complement-related protein), GBP28 (Gelatin-binding protein), adipoQ, apM1 (Adipose most abundant gene transcript 1) [1, 2]. Adiponectin is an adipocyte-secreted hormone, consisting of 244 amino acids with a molecular weight of approximately 30kDa (28-30kDa). It is one of the most abundant proteins in human blood, with circulating concentrations of 0.5-30 µg/ml, which accounts for approximately 0,01% of total plasma protein [2].

The protein consists of four domains: one globular C-terminal, one collagen-like N-terminal, one signaling peptide and one hyper variable domain. The globular domain has significant sequence and structural similarities to the complement factor C1q [2,3]. The globular domain also has structural similarities to TNF-α [3-5].

Adiponectin concentration is reversely associated with type 2 diabetes, coronary artery disease and obesity, all together called the metabolic syndrome. Adiponectin decreases blood glucose and free fatty acid serum concentrations and increases insulin sensitivity [7]. Adiponectin has also been shown to have anti-inflammatory effects [2].

Adiponectin has been suggested to exist in different forms in circulation: monomers, isolated globular forms (the globular domains), trimers, hexamers and larger oligomers [8-11]. Monomers are believed to associate in circulation to trimers through the globular domains. Trimers are associated to larger oligomers through the collagen-like domain [7].

However, recent studies indicate that adiponectin may not be present in circulation as monomers or isolated globular forms, but rather in multimeric structures. The studies have shown that the dominant forms of adiponectin that circulates in human blood are hexamers (LMW) and lager oligomers (HMW) [6, 12-14]. The LMW adiponectin levels does not seem to differ between insulin sensitive- and insulin resistant subjects, nor does LMW adiponectin differ between men and women. The increased levels of total adiponectin in insulin sensitive subjects and women were caused by increased amounts of HMW adiponectin. Both total and HMW adiponectin showed significant differences between the insulin sensitive- and insulin resistant subjects according to Lara-Castro et al. 2006 [6].

Several isoforms of adiponectin do circulate in blood. It is yet to be determined whether all isoforms are secreted by the adipocytes, whether there is a posttranscriptional assembly of HMW adiponectin in blood or whether the HMW form is secreted and degraded in blood. The individual metabolic significance of each adiponectin isoform also remains unclear [6].

PRINCIPLE OF THE PROCEDURE

Mercodia Adiponectin ELISA is a solid phase two-site enzyme immunoassay. It is based on the sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the adiponectin molecule. During incubation, adiponectin in the sample react with anti-adiponectin antibodies bound to microtitration well. After washing, peroxidase conjugated anti-adiponectin antibodies are added and after the second incubation and a simple washing step that removes unbounded enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

WARNINGS AND PRECAUTIONS

- For research use only. Not for use in diagnostic procedures. Not for internal or external use in humans or animals.
- The content of this kit and their residues must not be allowed to come into contact with ruminating animal or swine.
- The Stop Solution in this kit contains 0.5 M H_2SO_4. Follow routine precautions for handling hazardous chemicals.
- All patient samples should be handled as capable of transmitting infections.

MATERIAL REQUIRED BUT NOT PROVIDED

- Pipettes for 20, 25, 50, 100, 200 and 1000 µl (repeat pipettes preferred for addition of enzyme conjugate solution, Substrate TMB and Stop Solution)
- Beakers and cylinders for reagent preparation
- Redistilled water
- Microplate reader (450 nm filter)
- Plate shaker (The recommended velocity is 700-900 cycles per minute, orbital movement)
- Microplate washing device

REAGENTS

Each Mercodia Adiponectin ELISA kit (10-1193-01) contains reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2-8°C.

Coated Plate (Mouse monoclonal anti-human adiponectin)	1 plate 8-well strips	96 wells	Ready for use
For unused microtitration strips, reseal the bag using adhesive tape and store at 2-8°C for two months.			
Calibrators 1, 2, 3, 4, 5 (Recombinant human adiponectin) Concentration stated on vial label. Color coded yellow	5 vials	1000 µl	Ready for Use
Calibrator 0 Color coded yellow	1 vial	5 ml	Ready for use
Assay Buffer Color coded red	1 vial	12 ml	Ready for use
Sample Buffer 2X Dilute with 50 ml redistilled water to make sample buffer. Color coded yellow Storage after dilution: 2-8°C for two months	1 bottle	50 ml	
Enzyme Conjugate 11X (Peroxidase conjugated mouse monoclonal anti-human adiponectin)	1 vial	1.3 ml	Preparation, see below
Enzyme Conjugate Buffer Color coded blue	1 vial	13 ml	Ready for use
Wash Buffer 21X Dilute with 800 ml redistilled water to make wash buffer. Storage after dilution: 2-8°C for two months	1 bottle	40 ml	
Substrate TMB (TMB) Colorless solution *Note! Light sensitive!*	1 bottle	22 ml	Ready for use
Stop Solution 0.5 M H_2SO_4	1 vial	7 ml	Ready for use

Preparation of enzyme conjugate solution

Prepare the needed volume of enzyme conjugate solution by dilution of Enzyme Conjugate 11X, (1+10) in Enzyme Conjugate Buffer according to the table below. Mix gently.
When preparing enzyme conjugate solution for the whole plate or if the reagents are to be used within two months, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X vial.

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate Buffer
12 strips	1 vial	1 vial
8 strips	700 µl	7 ml
6 strips	500 µl	5 ml
4 strips	400 µl	4 ml

Storage after dilution: 2-8°C for two months.

SPECIMEN COLLECTION AND HANDLING

Serum

Collect blood by venipuncture, allow to clot. Separate the serum by centrifugation at 4 300 g for 15 minutes at 2-8°C. Specimen can be stored at 2-8°C up to 14 days. For longer periods, store samples at –20°C. Avoid repeated freezing and thawing.

Plasma

Collect blood by venipuncture into tubes containing heparin, citrate or EDTA as anticoagulant, and separate the plasma fraction. Samples can be stored at 2-8°C up to 14 days. For longer periods store samples at –20°C. Avoid repeated freezing and thawing.

PREPARATION OF SAMPLES

Samples should be diluted 1/101 v/v with sample buffer (20 µl sample + 2,0 ml sample buffer). Diluted samples can be stored at 2-8°C up to 14 days. *Note!* Buffers containing sodium azide (NaN_3) can not be used for sample dilution.

TEST PROCEDURE

All reagents and samples must be brought to room temperature before use. Prepare a calibrator curve for each assay run.

1. Prepare enzyme conjugate solution (according to the table on previous page), sample buffer, wash buffer and samples.
2. Prepare sufficient microplate wells to accommodate Calibrators and samples in duplicate.
3. Pipette 25 µl each of Calibrators and samples into appropriate wells.
4. Add 100 µl of Assay Buffer into each well.
5. Incubate on a plate shaker (700-900 rpm) for 1 hour at room temperature (18-25°C).
6. Wash plate 6 times with automatic plate washer
   or
   aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.
7. Add 100 µl of enzyme conjugate solution into each well.
8. Incubate on a plate shaker for 1 hour at room temperature (18-25°C).
9. Wash plate 6 times with automatic plate washer
   or
   aspirate the reaction volume completely and fill each well with 350 µl wash buffer. Aspirate liquid completely. Repeat 5 times. After final wash, invert and tap the plate firmly against absorbent paper.
10. Add 200 µl Substrate TMB into each well.
11. Incubate for 15 minutes at room temperature (18-25°C).
12. Add 50 µl Stop Solution to each well.
    Place the plate on the shaker for approximately 5 seconds to ensure mixing.
13. Read optical density at 450 nm and calculate results.
    Read within 30 minutes.

Note! To prevent contamination between the conjugate and substrate, separate pipettes are recommended.

INTERNAL QUALITY CONTROL

Commercial controls such as Mercodia Obesity Control kit (10-1241-01) and/or internal serum pools with low, intermediate and high adiponectin concentrations should routinely be assayed as unknowns, and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number; reconstitution dates of kit components; OD values for the blank (sample buffer), Calibrators and controls.

CALCULATION OF RESULTS

Computerized calculation

The concentration of adiponectin is obtained by computerized data reduction of the absorbance for the Calibrators 1-5 versus the concentration using cubic spline regression.

Manual calculation

1. Plot the absorbance values obtained for the Calibrators 1-5 against the adiponectin concentration on a log log paper and construct a calibrator curve.
2. Read the concentration of the unknown samples from the calibrator curve.
3. Multiply the concentration with the dilution factor.

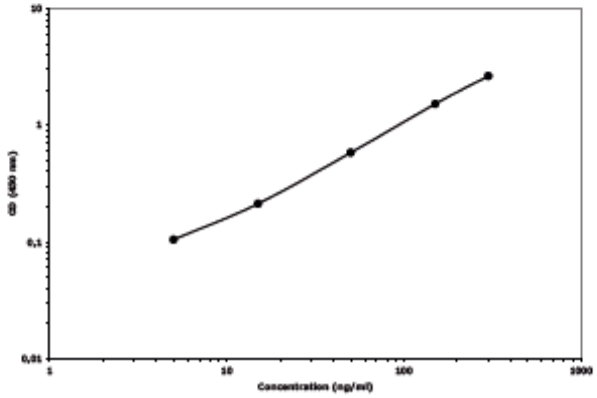
Example of results

Wells	Identity	A_{450}	Mean conc. ng/ml	x101 µg/ml
1A-B	Calibrator 0	0.059/0.056		
1C-D	Calibrator 1 (5 ng/ml)*	0.106/0.102		
1E-F	Calibrator 2 (15 ng/ml)*	0.207/0.216		
1G-H	Calibrator 3 (50 ng/ml)*	0.563/0.559		
2A-B	Calibrator 4 (150 ng/ml)*	1.477/1.567		
2C-D	Calibrator 5 (300 ng/ml)*	2.602/2.681		
2E-F	Unknown 1	0.374/0.367	29.825	3.012
2G-H	Unknown 2	0.754/0.758	67.178	6.785
3A-B	Unknown 3	1.385/1.373	133.340	13.467

*Exact concentration indicated on vial label.

Example of calibrator curve

A typical calibrator curve is shown here. Do not use this curve to determine actual assay results.



LIMITATIONS OF THE PROCEDURE

As with all diagnostic tests, a definitive diagnosis should not be based on the results of a single test, but should be made by a physician after all clinical findings have been evaluated.
Grossly lipemic, icteric or haemolyzed samples do not interfere in the assay.

EXPECTED VALUES

Good practice dictates that each laboratory establishes its own expected range of values.

Mercodia Adiponectin ELISA detects LMW(Hexamer 230kDa) and HMW(Oligomer >420 kDa) adiponectin, as determined by size exclusion gel chromatography.

The different multimeric forms of endogenous adiponectin were studied and separated in serum from a healthy individual by a three-step method; ammonium sulphate precipitation followed by ion exchange and gel filtration chromatography.

With ion exchange chromatography, proteins binds to the matrix with electrostatic forces causing separation since different proteins/isoforms have different total net charges or isoelectric points. The ion exchange column used was Mono Q 10/100GL (GE Healthcare). Triethanolamine buffer was used for eluting the proteins.

The isoforms of adiponectin has been shown to have different isoelectric points and post-translational patterns [15]. Proline hydroxylation and lysine hydroxylation/glycosylation are believed to have great importance on the assembly of the oligomers [12,15].

Five clearly distinct peaks were visible when the serum adiponectin was separated by ion exchange chromatography, indicating that the serum adiponectin analyzed presents at least five different post-translational patterns, yielding different isoelectric points, see figure 1 below. Pools A-E were further analyzed by size exclusion gel chromatography to determine the apparent size of the multimeric adiponectin forms.

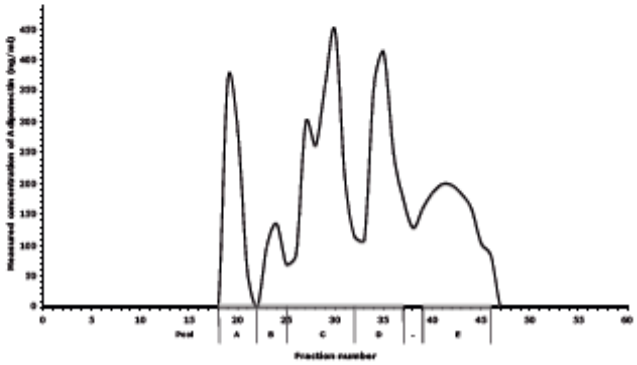


Figure 1 Elution profile of serum adiponectin from ion exchange chromatography and as identified by the Mercodia Adiponectin ELISA. Peak fractions were pooled to A, B, C, D and E.

Size exclusion gel filtration chromatography separates proteins according to apparent globular size. The gel filtration column that was used was HiLoad 16/60 Superdex 200 prep grade (GE Health care). PBS was used for eluting the proteins.

Three dominant multimeric forms were visible when the serum adiponectin was seperated by size exclusion gel chromatography, with apparent sizes of 230 kDa, 420 kDa and > 600 kDa respectively, and interpreted as LMW (hexamer 230 kDa) and HMW (420 kDa and >600 kDA), see figure 2 below.

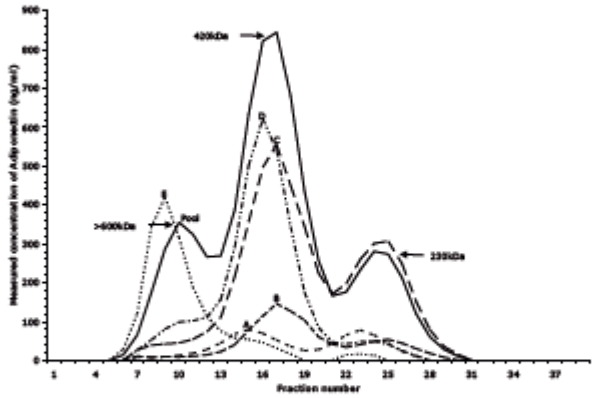


Figure 2 Elution profile of serum adiponectin from size exclusion gel filtration chromatography and as identified by the Mercodia Adiponectin ELISA. Each pool (A,B, C, D and E) was analyzed separately and together as one pool (Pool).

In conclusion, the serum adiponectin analyzed displayed three dominant multimeric forms based on size, and five different forms based on isoelectric points, or total net charges.

PERFORMANCE CHARACTERISTICS

Detection limit

Detection limit is defined as the Capability of Detection according to ISO11843-Part 1. Capability of Detection should be seen as part of a method validation, rather than the lowest concentration that can be measured.

The detection limit is 1.25 (ng/ml) as determined by the methodology described in ISO11843-Part 4.

Concentration of samples with absorbance below Calibrator 1 should not be calculated, instead expressed as less or equal to (≤) the concentration indicated on the vial for Calibrator 1.

Recovery

Recovery upon addition is 92-109% (mean 101%).
Recovery upon dilution is 89-111% (mean 98%).

Hook effect

There is no existing hook effect.

Precision

Each sample was analyzed in 4 replicates on 39 different occasions.

Sample	Mean value (ng/ml)	Coefficient of variation		
		within assay %	between assay %	total assay %
1	29.7	3.0	5.3	5.5
2	65.9	2.7	5.0	5.2
3	13.0	3.0	5.8	6.0

Specificity

The following crossreactions have been found:

C1q	≤ 0.007%
TNF-α	n.s

CALIBRATION

Adiponectin ELISA is calibrated against a highly purified, fully validated, commercial adiponectin preparation. The concentration of adiponectin is expressed in ng/ml.

WARRANTY

The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by Mercodia AB may affect the results, in which event Mercodia AB disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use. Mercodia AB and its authorized distributors, in such event, shall not be liable for damages indirect of consequential.

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Immunoassay Kits Beyond The Ordinary

Adiponectin (Multimeric) EIA

For the quanitative, selective determination of High Molecular Weight (HMW), Mid-Molecular Weight (MMW) and Total Adiponectin in human serum or plasma.

For Research Use Only. Not For Use In Diagnostic Procedures.

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Storage:	2-10°C
Version:	03/13/05 – ALPCO_02/08/07

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APPLICATION

This assay is intended for the quantitative, selective determination of High Molecular Weight (HMW), Mid-Molecular Weight (MMW) and Total Adiponectin in human serum or plasma.

INTRODUCTION

Adiponectin is a 244 amino acid protein (one of several known adipocytokines) secreted by the adipocyte. It has been reported that adiponectin circulates in the blood in various oligomeric complexes consisting of multiple proteins bound together. These complexes range from dimeric forms to a "bouquet" structure of 9 or more proteins. [1-3] Adiponectin has been reported to have several physiological actions, such as protective activities against atherosclerosis, improvement of insulin sensitivity, and prevention of hepatic fibrosis. In recent years, the relationship of these physiological actions with the circulating multimer structure of adiponectin has been attracting wide attention. It has been reported recently that the ratio of high-molecular weight adiponectin in circulation to total adiponectin level of the subject reflects the condition of disease more clearly than total adiponectin levels alone.[4-6]

The structure of adiponectin multimers was elucidated recently by selectively separating each adiponectin multimer from human plasma. This led to the discovery of an albumin-binding trimer, in addition to the already documented trimer, hexamer and HMW form. Following a further report that part of the multimer form is able to be digested selectively by a certain protease, this kit utilizes a method of measurement incorporating pretreatment with proteases for selective measurement of human multimeric adiponectin.[7] In addition, in order to provide a reference for total adiponectin levels, a new, simple pretreatment method is utilized. By the proposed method, multimeric adiponectin in the serum is converted mainly to a dimer via the addition of an SDS-containing acid buffer (without boiling step). [8]

ASSAY PRINCIPLES

This kit operates on the principle of a "sandwich" format enzyme-linked immunosorbent assay (ELISA). The specific antibodies used in the kit are anti-human adiponectin monoclonal antibodies (MoAbs) directed to two independent epitopes. The specimens are pre-treated as described below, and total adiponectin and individual multimers of adiponectin are determined selectively, directly or indirectly. Multimers of adiponectin are classified into four fractions with this kit:

1) Total adiponectin fraction: "Total-Ad"-assayed directly on the plate
2) High-molecular adiponectin fraction (equivalent of dodecamer -octodecamer): "HMW-Ad"-assayed directly on the plate
3) Middle-molecular adiponectin fraction (equivalent of hexamer): "MMW-Ad"-inferred value obtained by subtracting the concentration of HMW-Ad from the combined concentration of MMW-Ad + HMW-Ad
4) Low-molecular adiponectin fraction (equivalent of trimer including albumin-binding adiponectin): "LMW-Ad"-inferred value obtained by subtracting the combined concentration of MMW-Ad + HMW-Ad from the total concentration of Ad.

The microtiter plate wells have been coated with an anti-human adiponectin monoclonal antibody. Adiponectin in the standards and pretreated specimens are captured by the antibody during the first incubation. Afterwards, a wash step removes all unbound material. Subsequently, an anti-human adiponectin antibody which has been biotin-labeled is added and binds to the immobilized adiponectin in the wells. After the second incubation and subsequent wash step, HRP–labeled streptavidin is added. After the third incubation and subsequent wash step, substrate solution is added. Finally, stop reagent is added after allowing the color to develop. The intensity of the color development is read by a microplate reader.

The absorbance value reported by the plate reader is proportional to the concentration of adiponectin in the sample.

In this kit, pre-treated normal human serum by sample pre-treatment buffer is used as the Calibrator. The sample Pre-Treatment procedure used in this assay is very important for good results. It is outlined here briefly, and explained further in the Assay Procedure section of this insert.

1) "Total-Ad" assay: Specimens are treated with SDS-containing acid buffer to convert multimeric adiponectin mainly to a dimer form.

2) For "HMW-Ad" assay: The protease that selectively digests LMW-Ad and MMW-Ad acts on specimens, and remaining MW-Ad fraction is treated with SDS-containing acid buffer to convert it to a dimer form. Upon addition of the SDS-buffer, the digestive reaction of protease is stopped.

3) For combined "MMW-Ad" and "HMW-Ad" assay: The protease that selectively digests only LMW-Ad acts on specimens, and remaining MMW-Ad and HMW-Ad are treated with SDS -containing acid buffer to convert these fractions to a dimer form. Upon addition of the SDS-buffer, the digestive reaction of protease is stopped.

KIT COMPONENTS

Reagent	Composition	Amount
Wash buffer concentrate	Phosphate buffer (pH 7.2)	100 ml x 1 vial
Sample pre-treatment buffer	Citrate buffer (pH 3.0) containing SDS	50 ml x 1 vial
Dilution buffer	Phosphate buffer (pH 7.2) containing BSA	100 ml x 1 vial
Monoclonal Ab coated plate	Anti-human Adiponectin mouse monoclonal Ab coated 96-well	1 plate
Calibrator	Human serum stablized in sample pre-treatment buffer	0.25 ml x 1 vial
Biotin Labeled MoAb	Biotin Conjugated anti-human adiponectin	6.0 mL x 1 vial
Enzyme-labeled streptavidin	Horse-radish peroxidase (HRP) 6.0 labeled streptavidin	ml x 1 vial
Substrate (lyophilized)	O-phenylenediamine	2 vials
Substrate buffer	Citrate buffer (pH 5.0) containing H_2O_2	15 ml x 1 vial
Stop reagent	7.7% H_2SO_4	15 ml x 1 vial
Protease I (lyophilized)	Protease	1 vial
Protease II (lyophilized)	Protease	1 vial
Protease buffer	Tris buffer (pH 8.0)	50 ml x 1 vial

REAGENT PREPARATION AND STORAGE

Proteases are extremely temperature sensitive. It is critical that all reagents must be allowed to reach room temperature prior to use.

a) Wash buffer
Dilute Wash buffer concentrate with 900mL purified water. Working Wash buffer should be stored at 2-10°C.

b) Sample pre-treatment buffer
A white precipitate may form in this vial. This precipitate will be completely dissolved by warming the solution to room temperature and thoroughly stirring it before use.

c) Dilution buffer
Dilution buffer is supplied ready-to-use.

d) Monoclonal-antibody coated wells
MoAb coated wells are supplied ready-to-use. The unused strips should be returned to the laminate bag and stored at 2-10° C.

e) Working Calibrator
Note: There may be a precipitate in the tube. Allow the solution to stand at room temperature and stir thoroughly before using. Avoid foaming.
Just prior to use, dilute the Calibrator 1:101 with Dilution buffer and further the serial dilution to create a standard curve:

**Standard number 1 is the Stock calibration material included with the kit, diluted 1:101. Actual concentration (diluted 1:101) is found on the vial label; subsequent standards must be calculated from this value.		
Standard number:	Calibration material to add:	Diluent to add:
1	150 µl of stock solution	0 µl
2	150 µl of standard 1	150 µl
3	150 µl of standard 2	150 µl
4	150 µl of standard 3	150 µl
5	150 µl of standard 4	150 µl
6	150 µl of standard 5	150 µl
7	150 µl of standard 6	150 µl
8	0	150 µl

* The remaining Calibrator should be stored at 2-10°C and the same procedure should be repeated when it is to be used again.
* Since precipitation is temporarily caused when Calibrator is added to Dilution buffer, stir it thoroughly.
* The working calibrator should be prepared at the same time as the dilution after specimen treatment and add the treated specimen and the working calibrator to the MoAb coated wells in succession.

f) Biotin labeled-MoAb
Biotin labeled-MoAb is supplied ready-to-use.

g) Enzyme labeled streptavidin
Enzyme labeled streptavidin solution is supplied ready-to-use.

h) Substrate solution
Just prior to use, reconstitute the Substrate (lyophilized) by adding 6 mL of substrate buffer to the substrate vial. The substrate solution should be used immediately after reconstitution and the remaining solution should be discarded.

i) Stop reagent
Stop reagent is used as it is.

j) Protease solution I
Reconstitute Protease I (lyophilized) by adding 10 mL of protease buffer to the vial and dissolve completely by mixing at room temperature for 1 5-30min. Protease I is stable for 2 days at 2-10°C. Freeze below -30°C for extended storage.

k) Protease solution II
Reconstitute Protease II (lyophilized) by adding 10 mL of protease buffer to the vial and dissolve completely by mixing at room temperature for 15-30 min. Protease II is stable for 2 days at 2-10°C. Freeze below -30°C for extended storage.

ASSAY PROCEDURE

1) Pretreatment of specimens
Each specimen may be treated by any combination of the pretreatment options below, but separate aliquots must be used for each option. For example, if HMW and total adiponectin concentrations are being quantified on the same sample, treat two aliquots of the sample: one by the "total-Ad" procedure and one by the "HMW-Ad" procedure.

~Pre-treatment option 1: For "Total-Ad" assay

Add **100 µl** of Protease Buffer (Tris buffer, pH 8.0) and **400** uL of Sample Pre-treatment Buffer to **10** uL of serum or plasma.
Stir thoroughly (sample dilution = 1:51).

~Pre-treatment option 2: For combined "MMW-Ad" and "HMW-Ad" assay

Add **100** uL of Protease Solution I to **10** uL of serum or plasma and incubate for 20 min at 37° C.
Immediately add **400** uL of Sample Pre-Treatment Buffer.
Stir thoroughly (sample dilution = 1:51).

~Pre-treatment option 3: For "HMW-Ad" assay Add **100** iL of the Protease Solution II to **10** uL of serum or

plasma and incubate for 20 min at 37° C. Immediately add **400** uL of the Sample Pre-Treatment Buffer. Stir thoroughly (sample dilution = 1:51).

2) Dilution of Pretreated specimens

Further dilute pre-treated specimens 1:101 as follows:

Add **10** uL of the pre-treated specimen obtained in Steps 1 to 3 (see "Pretreatment of Specimens") to 1.0 ml Dilution Buffer (Phosphate Buffer + BSA, pH 7.2). **FINAL Sample dilution = 1:5151.**

*** Since precipitation is temporarily caused when a pretreated specimen is added to Dilution buffer, stir thoroughly.**

3) Assay Method

1) Plan your plate configuration. Determine how many strips will be needed and remove the excess strips from the microtiter plate. Store the remaining strips in the laminate bag.
2) Add 50 uL each of the working calibrator and diluted samples to the appropriate wells, according to the plate configuration.
3) Cover the plate with a plate sealer and incubate for 1 hour at room temperature (20-30 °C).
4) Decant the plate and strike the plate against absorbent towels to remove any excess liquid. Do not introduce absorbent materials into the wells! Wash by adding 350-400 uL of Wash Buffer to each well (using a laboratory squeeze bottle, wash manifold, or automated plate washer); decant wash buffer and strike plate against absorbent towels to remove residual liquid. Repeat this cycle twice, for a total of 3 washes.
5) Add 50 uL of Biotin-labeled monoclonal antibodies to each well. Cover the plate with a plate sealer and incubate for 1 hour at room temperature (20-30 °C).
6) Repeat wash step as described in Step 4.
7) Add 50 uL of the Enzyme-labeled streptavidin to each well. Cover the plate with a plate sealer and incubate for 30 min at room temperature (20-30 °C).
8) Repeat wash step as described in Step 4.
9) Add 50 uL of the Substrate solution to each well. Protect the plate from light and incubate for 10 min. at room temperature (20-30 °C).
10) Add 50 uL of the Stop solution to each well.
11) Using a microtiter plate reader set to 492 nm, read the absorbance of each well. Use a reference wavelength of 600-700 nm if desired.

Proteases are extremely temperature sensitive. It is critical that all reagents must be allowed to reach room temperature prior to use.

4) Calculations

Calculate the Ā absorbance by subtracting the absorbance of the 0 ng/mL calibrator from those of other calibrators and diluted samples. Plot the Ā absorbance of calibrators against the calibrator concentration on log-log or semi-log graph paper. Draw a smooth curve through these points to construct the calibration curve. Read the concentrations for the Diluted samples from the calibration curve. Calculate the concentration for the Diluted samples by multiplying by dilution factor (1:5151). Each fraction of Adiponectin multimers are calculates as follows.

- "Total-Ad": Concentration of the specimen prepared by Pre-treatment option 1.
- "HMW-Ad": Concentration of the specimen prepared by Pre-treatment option 3.
- "MMW-Ad": Concentration of the specimen prepared by Pre-treatment option 2 (HMW + MMW Ad) minus the concentration of the specimen prepared by Pre-treatment option 3 (HMW-Ad)
- "LMW-Ad": Concentration of the specimen prepared by Pre-treatment option 1 (Total-Ad) minus the concentration of the specimen prepared by Pre-treatment option 2 (HMW + MMW Ad).

PROCEDURAL NOTES

Proteases are extremely temperature sensitive. It is critical that all reagents must be allowed to reach room temperature prior to use.

1. This kit has been validated for analyzing human serum, EDTA-plasma or heparinized plasma. Citrated

plasma must not be used due to unqualified lower results.

2. Measurements of different adiponectin species of the same serum or plasma samples **MUST** be in the same plate. **DO NOT** calculate results between different plates.
3. A calibration curve must be run with each assay. Calibrators and samples should be assayed in duplicate.
4. If the concentration of adiponectin in a specimen exceeds the highest point of the calibration curve range, dilute pretreated specimen further with Dilution Buffer and re-assay.
5. Observe all specified reaction times and temperatures outlined in this manual. These parameters are especially important while pre-treating samples with proteases.
6. If the kit is not used entirely during the premier run, the remaining reagents may be stored as directed in the package insert and used one subsequent time before the expiration date.
7. Samples must be run promptly after the step of addition of the Sample pre-treatment buffer and added to the MoAb coated wells.
8. Remove all residual liquid completely after each step of the wash procedure.
9. Do not allow the wells to dry out or be damaged during the washing procedure.
10. Avoid carrying out this procedure in direct sunlight.

PERFORMANCE CHARACTERISTICS

1. Sensitivity
   The absorbance is not less than 0.9 OD when the standard solution (4.8 ng/mL) is measured.
2. Specificity
   The observed value is 80-120% of the known concentration when a control specimen of known concentration is used.
3. Reproducibility
   The coefficient of variation for observed values is not more than 15% when the same specimens are measured simultaneously 8 times.
4. Range of measurement:
   This test kit is effective in the range of 0.075 to 4.8 ng/mL when operated as directed under the section entitled "Assay Procedure".

WARNINGS AND PRECAUTIONS

1. The human serum contained in the calibrator was tested and found negative for presence of the HBs antigen, HIV antibody, and HCV antibody, however all specimens should be handled carefully as though capable of transmitting infection.
2. Stop reagent (7.7% H_2SO_4) is hazardous and can cause severe burns. In case of eye contact, rinse immediately with plenty of water, and seek medical advice. In case of contact with skin or clothing, rinse immediately with plenty of water.

STORAGE OF REAGENTS

The kit reagents should be stored at 2-10°C. **DO NOT FREEZE.**

EXPIRATION DATE Indicated on the package.

NUMBER OF ASSAYS 96 tests per kit

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7) International Publication Number WO 2005/038457
8) International Publication Number WO 2005/038458

Manufactured By: Daiichi Pure Chemical Toyko, Japan

Appendix 4
Papers

- ***High Molecular Weight Adiponectin Is Not Associated with Incident Coronary Heart Disease in Older Women: A Nested Prospective Case-Control Study***
  Naveed Sattar, Pauline Watt, Lynne Cherry, Shah Ebrahim, George Davey Smith, and Debbie A. Lawlor

- ***Serial changes in adiponectin and BNP in ACS patients: paradoxical associations with each other and with prognosis***
  Donald S. C. Ang, Paul Welsh, Pauline Watt, Scott M. Nelson, Allan Struthers and Naveed Sattar.

High Molecular Weight Adiponectin Is Not Associated with Incident Coronary Heart Disease in Older Women: A Nested Prospective Case-Control Study

Naveed Sattar, Pauline Watt, Lynne Cherry, Shah Ebrahim, George Davey Smith, and Debbie A. Lawlor

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Context: Adiponectin levels appear weakly linked to incident vascular disease, but the high molecular weight (HMW) fraction may be more relevant.

Objective: Our objective was to test whether HMW adiponectin, the key biologically active fraction, is linked to incident coronary heart disease (CHD) events.

Design, Participants, and Main Outcome Measures: We assessed the association between HMW adiponectin (measured by ELISA) and CHD risk in a prospective (4-yr) case-control study nested within the British Women's Heart and Health Study. All women were postmenopausal.

Setting: Women were seen in a primary care setting.

Results: Among both cases (n = 167) and controls (n = 333), HMW adiponectin positively correlated with age and high-density lipoprotein cholesterol and inversely correlated with waist to hip ratio, fasting insulin, fasting glucose, homeostasis model assessment for insulin resistance scores, C-reactive protein, and triglycerides, in similar fashion to total adiponectin. The age-adjusted relative risk ratio for a doubling of HMW adiponectin was 0.96 (95% confidence interval, 0.78–1.18), and adjustment for any of the potential confounding or mediating variables did not substantively alter this. Additional adjustments for childhood social class, alcohol consumption, hormone replacement therapy use, statin, aspirin, or blood pressure medication did not alter the null association. When we examined the effect of HMW adiponectin by quarters of its distribution, there was no evidence of any associations (P trend = 0.71). There was also no association of the ratio of HMW adiponectin to total adiponectin with CHD risk; age-adjusted relative risk per doubling of the ratio was 1.10 (95% confidence interval, 0.80–1.50).

Conclusions: Despite associations with total adiponectin and insulin resistance, our data go against any apparent association between HMW adiponectin levels and incident CHD events. ***J Clin Endocrinol Metab* 93: 1846–1849, 2008)**

High levels of adiponectin are unequivocally associated with lower risk of incident diabetes (1–3). High adiponectin was initially reported to be independently associated with lower risk for incident myocardial events (4). Consequently, its role in the common soil hypothesis was proposed. However, subsequent studies, inclusive of the British Regional Heart Study as well as a metaanalysis of seven prospective studies involving a total of 1318 coronary heart disease (CHD) cases, did not confirm a significant association of adiponectin with risk for incident CHD events [odds ratio for top third *vs.* bottom, 0.84; 95%

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Abbreviations: BWHHS, British Women's Heart and Health Study; CHD, coronary heart disease; CI, confidence interval; CRP, C-reactive protein; HDL-C, high-density lipoprotein-cholesterol; HMW, high molecular weight; HOMA-IR, homeostasis model assessment for insulin resistance; HRT, hormone replacement therapy; LDL, low-density lipoprotein-cholesterol.

confidence interval (CI), 0.70–1.01] (5). Nevertheless, because the high molecular weight (HMW) form of adiponectin appears to be 1) better associated with insulin sensitivity than total adiponectin (6), 2) more strongly associated with lower risk for incident diabetes (7), and 3) more strongly linked to protection against endothelial cell apoptosis (8), there is a need to examine whether this fraction is related to risk for vascular events.

Using a nested case-control study of the British Women's Heart and Health Study (BWHHS), we tested the hypothesis that higher levels of the HMW rather than total adiponectin (9) would be associated with lower risk of incident CHD events.

Subjects and Methods

The BWHHS is a prospective cohort study of 4286 women randomly selected from 23 British towns between 1999 and 2001 and who were aged 60–79 yr at baseline (10). The prospective case-control study and endpoint ascertainment nested within this cohort and used herein has been previously described (9). Briefly, an incident case was defined as either 1) death with an underlying or contributing cause of CHD (ICD10 codes I20-I25, I51.6) or 2) a myocardial infarction (World Health Organization criteria), first diagnosis of angina, or coronary artery bypass or angioplasty. There were 167 incident cases of CHD (101 nonfatal and 66 fatal). Two controls were randomly selected, within 5-yr age groups of the cases, from women without CHD at the baseline assessment. The BWHHS has local and multicenter research ethics committee approvals, and all participants provided written informed consent for their medical records to be reviewed.

Baseline blood samples were taken after a minimum 6-h fast. HMW adiponectin was measured by an ELISA (ALPCO Diagnostics, Salem, NH) with a protease to digest both the low and medium molecular weight forms, leaving the HMW form intact for measurement (11). This ELISA has been validated against conventional fractionation, with excellent correlations ($r = 0.94–0.97$) between methods (http://www.alpco.com/pdfs/47/Human%20Multimeric%20Adiponectin%20Details.pdf). The intraassay coefficient of variation was 6%. Because we had previously measured total adiponectin in the same cohort by a different ELISA (R&D Systems, Abingdon, UK) (9), we correlated total adiponectin by the ALPCO and R&D ELISAs in a subset of 80 samples; an excellent correlation of $r = 0.89$ between methods was noted despite blinded measurements, conducted several months apart with different dilutions. Repeated-sample freeze-thaw did not influence HMW adiponectin values.

Methods for insulin, glucose, and lipids levels and C-reactive protein (CRP) and homeostasis model assessment of insulin resistance (HOMA-IR) have been detailed previously (10). All blood samples were taken between 0800 and 1800 h with the time of sampling (to nearest minute) recorded. Levels of total adiponectin and HMW adiponectin did not vary by time of day of sampling.

Medication history, blood pressure, body mass index, waist and hip circumference, and information on adult and childhood occupational social class, smoking, alcohol consumption, and physical activity were determined as previously described (10).

Statistical analyses

Spearman's rank correlation coefficients were used to assess the associations of continuous covariables with HMW adiponectin and total adiponectin. There was no statistical evidence that any of these associations differed by case-control status, and therefore correlation coefficients are presented for the whole sample (cases and controls combined). Distributions of HMW adiponectin, total adiponectin, and other characteristics are presented for cases and controls. Differences between cases and controls were assessed using an unpaired t test for continuous variables and χ^2 test for categorical variables. Multiple logistic regression was used to assess the association of HMW adiponectin with CHD with adjustment for covariables. Geometric means and their 95% CI were used for positively skewed variables (HMW adiponectin, total adiponectin, CRP, glucose, insulin, HOMA-IR scores, and triglycerides) with logged values used in regression models. HMW adiponectin and total adiponectin were entered as continuous variables (natural logs of their levels) in these regression models. Because regression coefficients for logged exposure variables are difficult to interpret, these effect estimates are expressed as the risk ratio of CHD for a doubling of HMW or total adiponectin, as in other prospective studies of the association of total adiponectin with CHD. Possible nonlinear associations were explored by entering quarters of the HMW adiponectin distribution first as a series of three indicator variables and then as a continuous score and computing a likelihood ratio test comparing these two nested models. In the nested case control study design, the odds ratio derived from logistic regression directly estimates the incidence rate ratio and hence the risk ratio (12, 13). We repeated our analyses using conditional logistic regression and found the same, although less precise, results. All analyses were conducted in Stata version 9.0 (Stata Corp., College Station, TX).

Results

One control did not have a baseline sample; thus, data were available for 167 cases and 333 controls. HMW adiponectin was correlated with total adiponectin ($r = 0.75$) (Table 1). Both were inversely associated with waist to hip ratio, fasting insulin, glucose, HOMA-IR, CRP, and triglyceride levels and positively associated with high-density lipoprotein cholesterol (HDL-C) levels. There was no strong statistical evidence of heterogeneity between the associations of either form of adiponectin with any of these traits (all P values > 0.3).

Geometric mean (95% CI) levels of HMW adiponectin were not different in cases [4.07 (3.64–4.55) μg/ml] *vs.* controls [4.17 (3.90–4.47) μg/ml]; $P = 0.7$. As previously reported (9), cases had higher fasting insulin, glucose, HOMA-IR, low-density lipoprotein-cholesterol (LDL-C) and lower HDL-C, but triglyceride levels and systolic and diastolic blood pressure were similar. However, the prevalence of hypertension and smoking was greater, and use of hormone replacement therapy (HRT) less at baseline in cases compared with controls.

HMW adiponectin levels were lower in women who were in manual, compared with nonmanual, social classes in adulthood, but there were no strong associations of smoking, physical activity, alcohol consumption, or statin, aspirin, or HRT use with HMW adiponectin levels (full data not shown).

In multivariable analyses, there was no association between HMW adiponectin and incident CHD (Table 2). The age-adjusted relative risk ratio for incident CHD for a doubling of HMW adiponectin was 0.96 (95% CI, 0.78–1.18), and adjustment for any of the potential confounding or mediating variables, including glucose, did not substantively alter this (Table 2). The same was true when we removed the 25 women with baseline diabetes (data not shown). Additional adjustments for childhood social class, alcohol consumption, HRT use, statin, aspirin, or blood pressure medication did not alter the null association (data not shown). When we examined the effect of HMW adiponectin by quarters of its distribution, there was no

TABLE 1. Correlation of HMW adiponectin and total adiponectin with measures of adiposity, insulin, glucose, and lipid levels in British women aged 60–79 yr (n = 500)

	Spearman's rank correlation coefficient with HMW adiponectin	P	Spearman's rank correlation coefficient with total adiponectin	P
HMW or total adiponectin	0.75	<0.001	0.75	<0.001
Age	0.08	0.07	0.13	0.004
Body mass index	−0.12	0.008	−0.20	<0.001
Waist-hip ratio	−0.26	<0.001	−0.32	<0.001
Insulin	−0.35	<0.001	−0.41	<0.001
Glucose	−0.11	0.01	−0.20	<0.001
HOMA-IR	−0.30	<0.001	−0.38	<0.001
CRP	−0.17	0.002	−0.20	<0.001
Total cholesterol	0.00	0.97	−0.02	0.51
LDL-C	0.01	0.84	−0.01	0.63
HDL-C	0.37	<0.001	0.44	<0.001
Triglycerides	−0.34	<0.001	−0.42	<0.001
Systolic blood pressure	−0.06	0.20	−0.05	0.36
Diastolic blood pressure	0.05	0.31	0.01	0.62

evidence of any linear or nonlinear associations (Fig. 1). There was also no association of the ratio of HMW adiponectin to total adiponectin with CHD risk; age-adjusted relative risk per doubling of the ratio was 1.10 (95% CI, 0.80–1.50).

Discussion

As with total adiponectin (9), we could find no evidence of any association of the HMW adiponectin (or its ratio to total adiponectin) with incident vascular events in this prospective study of older British women. This finding further supports our previous contention (5) that circulating concentrations of adiponectin (and its fractions) relate strongly to diabetes but not to vascular event risk.

Several aspects of our study merit discussion. First, our method of assessment of HMW adiponectin is relatively new but robust. Although Bluher and colleagues (14) questioned the robustness of this method for measurement of HMW adiponectin, their study had only 60 women spanning glycemia thresholds and reported no significant association of total adiponectin by the ALPCO method with two other methods for total adiponectin (both r = 0.25; *P* value only described as nonsignificant in paper) and also no association of either total or HMW fractions with fasting insulin (r = −0.09 and r = −0.04, respectively, both nonsignificant). By contrast, in our study of 500 women predominantly without diabetes, not only did the HMW fraction correlate with total adiponectin ($r = 0.75$; $P < 0.001$) by a different method, the total adiponectin method by ALPCO correlated with the R&D ELISA (r = 0.89; $P < 0.001$) in a subset of 80 samples. In addition, the HMW fraction correlated with both fasting insulin (r = −0.35; $P < 0.001$) and with other metabolic parameters in the expected directions of association. Others have reported similar correlations between total and HMW adiponectin; Halperin *et al.* (15) reported a correlation of r = 0.72 between percent HMW and total adiponectin using the velocity sedimentation analysis method. Finally, the ALPCO HMW method has been previously validated against the more conventional methods employed for adiponectin fractionation (see *Subjects and Methods*) and has been shown by others (6, 16) to perform well and correlate to insulin sensitivity and related parameters, at least as well as total adiponectin, in larger sized cross-sectional studies than that published by Bluher *et al.* (14). Thus, methodological issues relating to the assay are unlikely to explain our null findings.

Interestingly, in line with our data, Halperin *et al.* noted no evidence of a relationship between total adiponectin or percent HMW and endothelium-dependent vasodilation (15), a vascular risk surrogate, in offspring of diabetic parents. Furthermore, higher adiponectin levels predict a higher, not lower, risk for cardiovascular disease mortality in the general population (17). HMW adiponectin also predicts higher risk of mortality in those with heart failure (18), an observation consistent with a positive correlation between circulating adiponectin and brain natri-

TABLE 2. Multivariable associations of HMW adiponectin with incident cases of CHD (n = 167 cases and 333 controls)

	Relative risk ratio (95% CI) for a doubling of HMW adiponectin	P linear trend	P nonlinear association
Model 1: age adjusted[a]	0.96 (0.78–1.18)	0.71	0.96
Model 2: confounder adjusted[b]	0.98 (0.79–1.21)	0.85	0.79
Model 3: adjusted for confounders and potential mediators[c]	1.01 (0.81–1.24)	0.89	0.88

[a] Model 1 was adjusted for age (continuous in years).

[b] Model 2 was the same as model 1 plus adjusted for adult social class (four-level categorical variable entered as a score-1 parameter) smoking (three-level categorical variable entered as a score-1 parameter), and physical activity (three-level categorical variable entered as a score-1 parameter).

[c] Model 3 was the same as model 2 plus adjusted for waist to hip ratio, systolic blood pressure, fasting levels of HDL, triglyceride (logged), glucose (logged), insulin (logged), and CRP (logged) (all continuous variables).

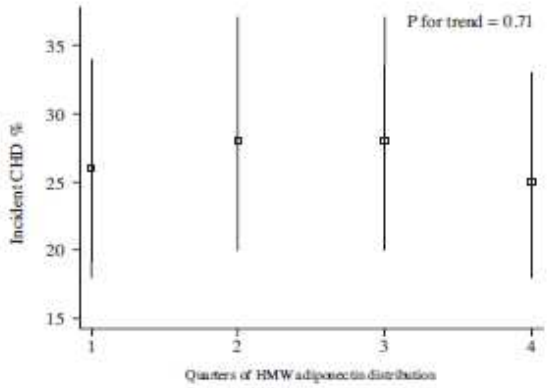

FIG. 1. Proportion (percentage) of women with incident CHD (cases) by quarters of the distribution of HMW adiponectin. The *vertical lines* represent 95% CI.

uretic peptide (19). These findings suggest that total and HMW adiponectin have complex relationships with vascular disease and that in any given population, some individuals have physiological (*i.e.* beneficial) elevations, whereas others have pathologically driven elevated levels (*i.e.* reflecting harmful signals). This suggestion provides a working hypothesis to explain paradoxical observations.

Our study has potential limitations. We used different assays for total (R&D) and HMW (ALPCO) adiponectin, but our observations above suggest this was an acceptable approach. Our results cannot be extrapolated necessarily to men or other ethnic groups. We accept that larger studies are needed to provide more reassurance of our null result, although the adjusted result of 1.01 (0.81–1.24) for incident CHD events with a doubling of HMW adiponectin is close to unity. Finally, although the correlation between HMW adiponectin and HOMA-IR score was not greater than between total adiponectin and HOMA-IR score, others have seen broadly similar results (20).

In conclusion, despite evidence to suggest HMW adiponectin may be the biologically active fraction of the adiponectin species, our results go against a strong inverse association between HMW adiponectin and incident CHD events. These findings further support a stronger association of adiponectin (and its fractions) with diabetes than vascular disease.

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Serial changes in adiponectin and BNP in ACS patients: paradoxical associations with each other and with prognosis

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ABSTRACT

Plasma adiponectin is inversely associated with the risk of coronary heart disease in healthy people. However, adiponectin and BNP (B-type natriuretic peptide) are both known to be positively associated with a risk of poor outcome, and with each other, in ACS (acute coronary syndrome) patients. Serial changes in plasma adiponectin and BNP following ACS have not been assessed previously, and may clarify these apparently paradoxical associations. In the present study, adiponectin, BNP, classical risk markers and clinical parameters were measured in plasma from 442 consecutive ACS patients in an urban teaching hospital, with repeat measures at 7 weeks ($n = 338$). Patients were followed-up for 10 months. Poor outcome was defined as mortality or readmission for ACS or congestive heart failure ($n = 90$). In unadjusted analysis, the change in adiponectin (but not baseline or 7-week adiponectin) was significantly associated with the risk of an adverse outcome {odds ratio (OR), 5.42 [95% CI (confidence interval), 2.78–10.55]}. This association persisted after adjusting for classical risk factors and clinical markers, but was fully attenuated by adjusting for the 7-week BNP measurement [OR, 1.13 (95% CI, 0.27–4.92)], which itself remained associated with risk [OR, 5.86 (95% CI, 1.04–32.94)]. Adiponectin and BNP positively correlated at baseline and 7 weeks, and the change in both parameters over 7 weeks also correlated ($r = 0.39$, $P < 0.001$). In conclusion, increases in plasma adiponectin (rather than absolute levels) after ACS are related to the risk of an adverse outcome, but this relationship is not independent of BNP levels. The results of the present study allude to a potential direct or indirect relationship between adiponectin and BNP post-ACS which requires further investigation.

INTRODUCTION

Adiponectin is a circulating adipokine which has been shown to have the unusual property of correlating inversely with markers of adiposity, including BMI (body mass index) and percentage body fat in epidemiological studies [1]. Adiponectin may have important metabolic regulatory functions [2], and it has also been suggested

Key words: acute coronary syndrome (ACS), adiponectin, B-type natriuretic peptide (BNP), heart failure, risk marker.

Abbreviations: ACS, acute coronary syndrome; ASE, American Society of Echocardiography; BMI, body mass index; BNP, B-type natriuretic peptide; CHD, coronary heart disease; CHF, congestive heart failure; CI, confidence interval; CV, coefficient of variation; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HF, heart failure; IQR, interquartile range; LV, left ventricular; LVEF, LV ejection fraction; LVH, LV hypertrophy; LVM, LV mass; LVMI, LVM index; LVSD, LV systolic dysfunction; MI, myocardial infarction; OR, odds ratio; STEMI, ST-elevation MI; NSTEMI, non-STEMI; TNFα, tumour necrosis factor α.

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that it may have some important anti-inflammatory properties [3]. In line with this, it may protect cardiac tissue and limit atherogenesis through inflammatory dampening mechanisms [4]. Experimental models in mice show that exogenous administration of adiponectin limits weight gain or even leads to weight loss [5]. In addition, overexpression of adiponectin in the *ApoE*$^{-/-}$ (where ApoE is apolipoprotein E) mouse model reduced atherogenesis [6].

Although plasma adiponectin is clearly linked to a lower risk for diabetes (as recently discussed in [7]), and an initial study linked low levels of adiponectin to an increased risk of CHD (coronary heart disease) events in healthy men [8], a previous meta-analysis suggested a more modest association with CHD [9]. Furthermore, studies in people with prevalent CHF [congestive HF (heart failure)] or ACS (acute coronary syndrome) show that high plasma levels of adiponectin are associated with greater disease severity [10,11] and with a higher risk of adverse outcome [12–15]. Thus circulating adiponectin concentrations may represent both a protective or harmful signal depending on the context.

BNP (B-type natriuretic peptide) and its inactive N-terminal metabolite NT-proBNP (released in a 1:1 ratio during processing) are sensitive markers of cardiac overload and are markers of prognosis in those with CHF [16]. Several studies have shown positive correlations of circulating baseline NT-proBNP and BNP with adiponectin in those with prevalent CHF and coronary artery disease [10–15,17,18]. Indeed, in the recent AtheroGene study [19], baseline adiponectin predicted the risk of a poor outcome in CHF independent of conventional risk factors, but not independent of BNP.

In order to examine further the potential pathological link between BNP and adiponectin, we investigated the inter-relationship of circulating adiponectin and BNP in 442 ACS patients both at baseline and at a 7-week follow-up (338 patients). Thus, for the first time, we examined the associations of all of these measures to the risk of adverse outcomes at 10 months to determine whether the change in either parameter was a better predictor of adverse events than a single measurement. We were also able to investigate whether a change in one over time correlated with a change in the other.

MATERIALS AND METHODS

Study population

A total of 442 Caucasian patients with a diagnosis of ACS (between August 2004 and November 2006) were consecutively recruited at Ninewells Hospital, Dundee, Scotland, U.K. Patients were recruited if they presented within 72 h after the onset of ischaemic discomfort and included those diagnosed with: (i) STEMI [ST-elevation MI (myocardial infarction)], ST elevation >1 mm in two limb leads or >2 mm in leads V1–V6 or new left bundle branch block; (ii) NSTEMI (non-STEMI), no ST elevation on ECG despite elevated troponin T > 0.01 μg/ml; and (iii) unstable angina, ischaemic chest pain lasting more than 30 min with no evidence of myocyte necrosis or ST elevation. Ethical approval was obtained from the Tayside Committee of Medical Research Ethics and all participating subjects gave written informed consent. The research was carried out in accordance with the Declaration of Helsinki.

During the ACS admission, the patients underwent the following clinical procedures: (i) clinical history and risk factor analysis; (ii) evaluation of admission Killip class defined as Killip class I (no clinical signs of HF), Killip class II (rales or crackles in the lungs, an S_3 gallop and elevated jugular venous pressure), Killip class III (frank acute pulmonary oedema) and Killip class IV [cardiogenic shock or hypotension (measured as systolic blood pressure lower than 90 mmHg), and evidence of peripheral vasoconstriction (oliguria, cyanosis or sweating)]; (iii) ECG, presence or absence of ST deviation (>1.0 mm); (iv) bedside BNP assay; (v) laboratory tests, admission haemoglobin, eGFR (estimated glomerular filtration rate) using the modification of diet in renal disease equation and serum troponin T levels; and (v) bedside echocardiography, LVSD [LV (left ventricular) systolic dysfunction] defined as LVEF (LV ejection fraction) < 45 % (using Simpson's biplane method) and LVH (LV hypertrophy) assessment.

Echocardiography

Transthoracic echocardiography was performed by one trained operator using an Acuson (Sequia 512) imaging system with a 3V2C transducer. The scan was performed with the patient lying in the left lateral position at approx. 45 %.

LVH assessment

Patients were studied with two-dimensional guided M-mode echocardiography in standard views. All measurements were made according to the ASE (American Society of Echocardiography) recommendation at end diastole, taken as the onset of QRS complex. The leading edge to leading edge convention was used to measure interventricular septal thickness, LV internal diameter and LV posterior wall thickness. Measurements were made over at least three separate cardiac cycles and the average was taken. LVM (LV mass) was calculated according to the formula of Devereux et al. [20] [0.80 (ASE LVM)+0.6] and was indexed to body surface area to give LVMI (LVM index). LVH was defined as LVMI greater than 95 g/m^2 in females and greater than 115 g/m^2 in males in accordance to ASE guidelines [21].

LV systolic function assessment

Quantitative assessment of LV systolic function was made using a modified Simpson's biplane method to calculate

an LVEF. Three measurements from successive cardiac cycles were made in the two-chamber and four-chamber views. LVSD was defined as an LVEF < 45 %.

Blood sample collection and analysis

Samples were collected by venipucture into EDTA vacutainer tubes or serum clot accelerators. One EDTA vacutainer of blood sample was kept at room temperature (18–25 °C) and was analysed for BNP within 4 h of the draw time. Whole blood was analysed with the triage BNP assay (Biosite) as reported previously [22]. The inter-assay CV (coefficient of variation) was 8.8 % at 71.3 pg/ml and 11.6 % at 4088 pg/ml. The detection limit was 5 pg/ml. An electrochemilumninescent immunoassay approved for quantitative measurement of troponin T was provided by Roche Diagnostics and run on a Roche Modular E170 unit. At 0.06 μg/l a CV of 10 % is achievable and the detection limit was 0.01 μg/l. Blood samples for adiponectin or other non-routine analyte measurements were spun at 2000 *g* for 15 min and the serum or plasma layers were aliquoted within 4 h, snap frozen, and stored at −80 °C. Total plasma adiponectin was analysed using a commercially available kit (R&D Systems). The inter-assay CV for the adiponectin assay was less than 8 %. The same methods were used for samples at follow-up measurements.

Follow-up measurements

At 7 weeks after baseline admission, an attempt was made to re-examine patients with a full ECG, transthoracic echocardiography to assess LVEF and routine blood tests including repeat bedside BNP and freezing of plasma samples (average time of follow-up was 52 ± 17 days). Of the 442 baseline patients (of whom 90 experienced adverse outcomes by 10 months), 433 were still alive and 338 consented to continuing participation in the study at 7 weeks (of whom 51 experienced adverse outcomes by 10 months). In relating BNP and adiponectin levels to the risk of an outcome we used all 442 baseline patients for whom measurements were obtained, and the 338 patient samples available to us from the 7-week measurement.

End points

The end point of death from any cause, readmission with ACS or admission with CHF was evaluated at 10 months (265.79 ± 80.31 days). Information on end points was collected from telephone interviews with patients or relatives of patients, hospital databases and patient case notes. The definition of readmission with ACS is as described above. CHF was defined as hospitalization for a clinical syndrome involving at least two of the following: paroxysmal nocturnal dyspnoea, orthopnoea, elevated jugular venous pressure, pulmonary crackles, third heart sound, and cardiomegaly or pulmonary oedema on chest X-ray. These clinical signs and symptoms must have represented a clear change from the normal clinical status, requiring intravenous diuretics, inotropic support or vasodilator therapy.

Statistical analysis

Continuous variables are summarized as medians and 25th–75th percentiles. For discrete variables, absolute and relative frequencies per category are given. Variables were logarithmically transformed to obtain normal distributions, with tertiles of adiponectin generated. Inter-group differences were assessed using a χ^2 test or ANOVA. Spearman correlation coefficients are reported. The association of adiponectin with adverse outcome was examined in different binary logistic regression models, first a univariate model, then in a model adjusting for classical risk factors, a third model additionally adjusted for baseline BNP and a fourth model for 7-week BNP. Goodness-of-fit of the models was tested by Hosmer–Lemeshow. Statistical significance was determined at $P < 0.05$.

RESULTS

Baseline characteristics

In the complete patient group the median BNP level at baseline was 154 [IQR (interquartile range), 53–336] pg/ml, and the median baseline adiponectin level was 6.76 (IQR, 4.16–10.82) μg/ml. With continuous analyses, the baseline correlation between BNP and adiponectin was $r = 0.32$ ($P < 0.001$).

Table 1 illustrates the baseline characteristics of the patients according to tertiles of adiponectin concentration. As expected, age increased across the tertiles of adiponectin (P for trend <0.001). Some other major cardiovascular risk factors showed inverse associations with adiponectin including the proportion of male gender ($P < 0.001$), BMI ($P < 0.001$) and total cholesterol ($P = 0.07$), whereas HDL (high-density lipoprotein)-cholesterol increased across tertiles ($P < 0.001$). Blood pressure, presence of hypertension, diabetes or smoking habit showed no significant association with adiponectin levels. Haemoglobin levels and kidney function (eGFR) decreased across adiponectin tertiles ($P < 0.001$). Among clinical measurements, LVEF ($P < 0.001$) decreased, and suboptimal prognosis according to Killip class increased ($P = 0.004$) with increasing adiponectin.

Changes in adiponectin and BNP from baseline to 7 weeks

The median BNP level at 7 weeks was 94 (IQR, 36–198) pg/ml and the median adiponectin level was 5.49 (IQR, 3.48–8.99) μg/ml. With continuous analyses the 7-week correlation between BNP and adiponectin was $r = 0.33$ ($P < 0.001$).

The overall change in adiponectin and BNP levels between baseline and 7-week follow-up for the whole

Table 1 Association between admission adiponectin levels (divided into thirds) and baseline characteristics of ACS patients ($n = 442$ with adiponectin measurement)

Values are numbers (%) or medians (25th–75th IQR) for continuous variables. The age is mean ± S.D. *P* values represent trends across adiponectin thirds. LBBB, left bundle branch block.

Characteristic	Bottom third adiponectin level (0.48–4.85 μg/ml; $n = 147$)	Middle third adiponectin level (4.86–8.94 μg/ml; $n = 148$)	Top third adiponectin level (8.95–35.40 μg/ml; $n = 147$)	*P* value*
Age (years)	60 ± 12	66 ± 11	69 ± 11	< 0.001
Gender (male/female) (*n*)	125/22 (85 %/15 %)	98/50 (66 %/34 %)	77/70 (52 %/48 %)	< 0.001
Systolic blood pressure (mmHg)	130 (115–150)	131 (118–150)	128 (110–147)	0.23
Diastolic blood pressure (mmHg)	70 (63–81)	72 (65–82)	70 (60–80)	0.18
Total cholesterol (mmol/l)	5.12 (4.19–6.08)	4.81 (3.94–5.95)	4.52 (3.85–5.62)	0.07
HDL cholesterol (mmol/l)	1.15 (0.99–1.33)	1.32 (1.11–1.47)	1.50 (1.2–1.82)	< 0.001
BMI (kg/m^2)	28.0 (26.0–31.0)	26.0 (24–29.0)	25.0 (22.0–29.0)	< 0.001
Smoking (*n*)				
Never	46 (31 %)	57 (38 %)	52 (35 %)	
Past	41 (28 %)	44 (30 %)	52 (35 %)	
Present	60 (41 %)	47 (32 %)	43 (30 %)	0.21
Haemoglobin (g/dl)	15.0 (14.1–15.8)	14.1 (12.7–14.9)	13.3 (12.1–14.4)	< 0.001
eGFR (ml·min^{-1}·1.73 m^{-2})	70 (59–78)	66 (52–75)	61 (51–73)	< 0.001
Troponin T (ng/ml)	0.27 (0.01–1.86)	0.36 (0.06–1.47)	0.31 (0.07–1.43)	0.98
Non ST-elevation ACS (*n*)	101 (69 %)	111 (75 %)	110 (75 %)	0.353
Type 2 diabetes (*n*)	21 (14 %)	23 (16 %)	16 (11 %)	0.48
History of hypertension (*n*)	61 (41 %)	71 (48 %)	74 (50 %)	0.29
ST deviation on ECG (*n*)	73 (50 %)	68 (46 %)	71 (48 %)	0.81
LBBB on ECG (*n*)	4 (2.7 %)	5 (3.4 %)	7 (4.8 %)	0.63
LVEF (%)	57 (49–63)	55 (45–62)	52 (39–61)	0.001
LVH (%)	84 (66 %)	81 (71 %)	74 (67 %)	0.680
Killip class II, III or IV (*n*)	8 (5 %)	13 (9 %)	25 (17 %)	0.004
BNP (pg/ml)	81 (39–216)	152 (44–343)	239 (114–489)	<0.001

patient group are shown in Figures 1a and 1b respectively. As can be seen from these Figures, both the change in adiponectin and BNP were near-normally distributed, with the former being slightly left-skewed and the latter right-skewed (skewedness statistic −1.22 and 1.80 respectively). For both biomarkers there was a spread of patients with either increases or decreases in either marker over the 7-week interval. Figure 2 (with both markers log-transformed to normality) shows a significant correlation between changes in BNP compared with the change in adiponectin. Indeed the correlation ($r = 0.39$, $P < 0.001$) was at least as strong as the baseline and 7-week correlations between the parameters noted above.

Associations of baseline adiponectin and BNP with adverse outcome

In unadjusted models, baseline adiponectin showed a borderline significant association with outcome {OR (odds ratio), 2.06 [95 % CI (confidence interval), 0.92–4.63]; $P = 0.08$} (Table 2a). Adjusting for classical risk factors (including clinical parameters) attenuated the association [OR, 1.63 (95 % CI, 0.54–4.93)]. Subsequent adjustments for BNP levels attenuated the association of baseline adiponectin with risk to near unity. However, as seen in Table 2(b), BNP remained significantly associated with poor outcome in every adjustment model, even after adjusting for baseline adiponectin [OR, 3.26 (95 % CI, 1.54–6.91)].

Associations of the 7-week adiponectin and BNP with adverse outcome

Among those who survived the first 7 weeks following ACS and who consented to further study, the association between 7-week adiponectin and the risk of a poor outcome was not considerably different to the association seen at baseline, and none was significant, perhaps due to reduced power with fewer observations (Table 2a). In contrast, the association of 7-week BNP with the risk of a poor outcome was substantially stronger, and was independent of classical risk factors and independent of 7-week adiponectin [OR, 6.84 (95 % CI, 2.54–18.45)] (Table 2b).

Associations of the change in adiponectin and BNP with adverse outcome

The change in adiponectin (Table 2a) appeared somewhat more strongly linked to the risk of an adverse outcome than either baseline or 7-week adiponectin. For the

Table 2 Association between baseline adiponectin/BNP, 7-week adiponectin/BNP, and change in adiponectin/BNP and collective adverse outcomes of mortality, HF or ACS ($n = 51$) within 10 months of index ACS events

Values are OR (95 % CI). ORs for adiponectin and BNP are for 1 log unit increase in the patient population. Classical risk factors: age, gender, BMI, pre-existing hypertension, pre-existing diabetes mellitus, Killip class II, III or IV, ST deviation, left bundle branch block, log troponin T on admission, chronic kidney disease stages 3, 4 or 5, LVSD, smoking status, haemoglobin on admission, and total cholesterol and HDL-cholesterol concentrations.

(a) Adiponectin

	Adiponectin measurement		
Adjustment model	Baseline adiponectin	7-Week adiponectin	Change in adiponectin
Unadjusted	2.06 (0.92–4.63)	2.19 (0.79–6.08)	5.42 (2.78–10.55)
Classical risk factors	1.63 (0.54–4.93)	1.31 (0.33–5.27)	3.99 (1.79–8.92)
Classical risk factors+baseline BNP	1.18 (0.38–3.64)	–	–
Classical risk factors+7-week BNP	–	0.74 (0.17–3.28)	–
Classical risk factors and change in BNP	–	–	3.17 (1.29–7.78)

(b) BNP

	BNP measurement		
Adjustment model	Baseline BNP	7-Week BNP	Change in BNP
Unadjusted	4.29 (2.59–7.11)	6.73 (3.33–13.60)	2.33 (1.13–4.82)
Classical risk factors	2.83 (1.35–5.92)	6.54 (2.49–17.21)	3.07 (1.25–7.53)
Classical risk factors+baseline adiponectin	3.26 (1.54–6.91)	–	–
Classical risk factors+7-week adiponectin	–	6.84 (2.54–18.45)	–

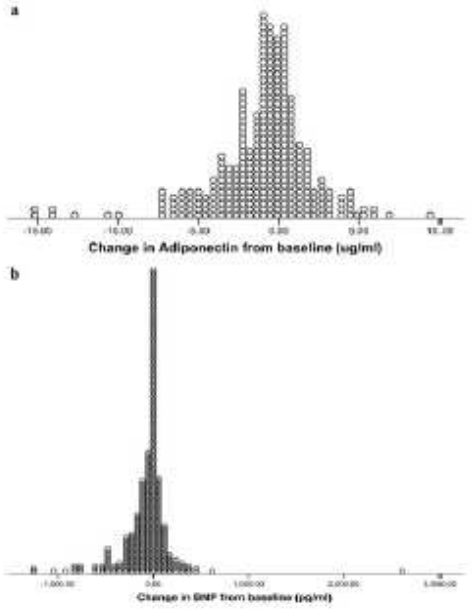

Figure 1 Untransformed overall change in adiponectin and BNP between baseline observations and follow-up observations 7 weeks later in the patient group

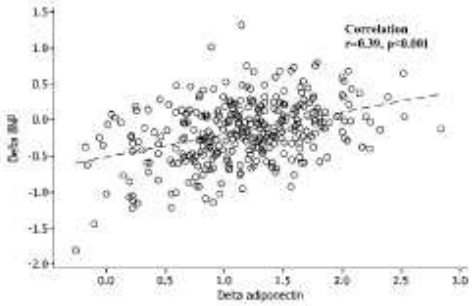

Figure 2 Correlation of the change from baseline at 7-weeks follow-up for adiponectin and BNP

Delta values represent (log of the follow-up value—log of the baseline value) for both adiponectin and BNP.

change in adiponectin, the unadjusted OR was 5.42 (95 % CI, 2.78–10.55), and attenuated to OR, 3.99 (95 % CI, 1.79–8.92) with adjustment for classical markers, and to OR, 3.17 (95 % CI, 1.29–7.78) with additional adjustment for the change in BNP. The change in BNP was not itself independently associated with adverse outcome in the latter model, suggesting that the change in adiponectin was the stronger marker.

Since the change in adiponectin and 7-week BNP appeared to have the strongest independent associations with outcome, we included both markers in the same multivariable model to test whether 7-week BNP could explain the association of the change in adiponectin with prognosis. After doing this, the change in adiponectin was no longer associated with the risk of an adverse outcome [OR, 1.13 (95 % CI, 0.27–4.92)], but the 7-week

BNP measurement remained significantly associated [OR, 5.90 (95 % CI, 1.04–32.94)].

DISCUSSION

To our knowledge, this is the first study to date to examine the association of circulating adiponectin and BNP with the risk of poor outcome in a group of ACS patients where serial measurements have been made. Importantly, an increase in adiponectin 7 weeks after admission was more strongly associated with the risk of an adverse outcome than a single measurement at either time point; in other words, if adiponectin levels increased, the adverse event risk was greater, whereas the absolute levels of adiponectin at either time point were less important. In contrast, the 7-week BNP measurement was more strongly associated with risk, superior to both baseline and change in BNP measurements. In terms of independent risk prediction, the strongest marker of the risk of adverse events was 7-week BNP, which was associated with risk independently of a change in adiponectin. Finally, although the patterns of risk associations of these markers are different, these results allow us to show that, following index admission for ACS, BNP and adiponectin associate with each other at baseline and at follow-up, and that a change in both markers also correlate ($r = 0.39$, $P < 0.001$).

The finding in the present study of a positive correlation between baseline circulating BNP and adiponectin, although apparently paradoxical on the basis of presumed functions of adiponectin, was an expected result based on previous findings in similar patient groups [10–15,17–19]. The present finding that 7-week BNP is more strongly related to adverse outcome than BNP at admission is in agreement with a recent study in 157 CHF patients, where discharge BNP was shown to be strongly associated with poor outcome [23].

We do not propose that the present results definitively answer the question of whether circulating BNP and adiponectin are or are not clinically useful in the prognosis of ACS. Rather, our results may give insight into the pathophysiology behind increased risk of ACS recurrence, CHF or death following an index ACS event. The results of the present study are suggestive of an interesting and potentially important pathphysiological pathway which links an increase in adiponectin to adverse prognosis in a manner not independent of BNP. Although an observational study cannot imply a causal link, it is interesting to speculate on the potential relationship of adiponectin and BNP following ACS.

We found that absolute levels of BNP at 7 weeks were the strongest predictor of prognosis. This makes sense, since levels of BNP following the settling of the acute-phase response and/or changes in clinical therapies after an event will better represent long-term cardiac/vascular status, and therefore subsequent risk. In contrast with BNP, increasing levels of adiponectin following index ACS admittance were potentially more strongly related to risk than baseline or 7-week adiponectin, and yet this relationship was dependent on 7-week BNP in multivariable models. This may be suggestive of some association between progressive disease severity (as measured by follow-up BNP) and changes in adiponectin. The AtheroGene investigators have recently shown that the relationship of baseline adiponectin with risk of death or non-fatal MI in a group of coronary artery disease patients is confounded by associations with BNP [19]. The results of the present study now extend this observation to show that the two parameters also change in parallel post-ACS, such that the risk associations of adiponectin with risk are dependent on BNP. This provides evidence that there may be a direct or indirect link between high post-ACS BNP and a rise in adiponectin in those at greatest risk of poor outcome.

If there is a link between the two parameters, are there any clues to mechanisms? Natriuretic peptides may directly stimulate higher adiponectin levels since a novel lipolytic and potential lipid-mobilizing effect of natriuretic peptides has been identified [24]. These actions appear to be mediated by specific adipocyte membrane receptors, which operate via a cGMP-dependent pathway and they may indirectly stimulate adiponectin production [24]. Furthermore, infusion of atrial natriuretic peptide (carperitide) in patients with HF leads to increased plasma adiponectin levels [25]. Alternatively, TNFα (tumour necrosis factor α) (among other pro-inflammatory cytokines) has been suggested to inhibit adiponectin expression in tissue from healthy subjects [26,27], but perhaps not in obese subjects [28]. TNFα is known to be elevated in conditions such as CHF and ACS [29], and hence, speculatively, the inhibitory signal may also be lost in such patients.

A recent attempt at explaining the physiology behind the reverse epidemiology of adiponectin in patients with ACS and CHF has been made, with the authors suggesting that elevated levels of adiponectin in these cases represent an attempt at counter-regulation of systemic inflammation [30]. Although this is a possibility, the additional possible direct cardioprotective and metabolic roles of adiponectin in ACS and CHF should not be overlooked. Importantly, adiponectin may directly reduce oxidative ischaemia/reperfusion injury [31–33], perhaps alluding to a mechanism whereby elevated BNP, in response to cardiac injury, induces an adiponectin-mediated cardioprotective response. Interestingly, this may also be true in silent ischaemia since others have suggested elevations in BNP may also occur in silent ischaemia [34,35], an observation potentially explaining why it is a better marker than simple echocardiography measures of cardiac dysfunction. Additionally, from a metabolic viewpoint, ACS and CHF are often considered to be insulin-resistant states, although individuals who are

insulin resistant without cardiovascular complications would be expected to have low levels of adiponectin. As we recently suggested [11], high levels of adiponectin in those with ACS and CHF may be a reflection of a salvage mechanism to improve insulin resistance and fatty acid oxidation, perhaps at a cost of cachexia in some cases. Regardless of mechanisms, greater cardiac disease severity may lead to a greater cardiological and metabolic salvage attempt and thus higher adiponectin levels. Alternatively, our speculation may be misguided and adiponectin may just be a passive marker of other physiological processes, its actions inhibited by peripheral 'adiponectin resistance'.

Limitations of the present study require consideration. The group of 442 ACS patients went on to have 90 end point events, and follow-up measurements were based on 51 events in 338 patients, which represents a relatively low power for the study and we cannot discount the possibility of type II errors. Nevertheless, our results on baseline associations of adiponectin with age and the female gender [36], as well as with BNP [10–15,17–19], and the link to end points [19,23,37], is consistent with the literature and suggest the results are externally valid. Further larger studies with serial measurements are required to confirm and expand on the potential mechanistic relationships of adiponectin, BNP and cardiac stress. In the present study we do not focus on the potential clinical utility of any measurement of adiponectin or BNP in the ACS clinical setting, but on pathophysiological changes in ACS which may require further clinical studies. Of additional interest, the use of genetics (e.g. Mendelian randomization) may help to tease out whether adiponectin is protective, harmful or passive for vascular risk for a variety of patient/cohort groups.

In conclusion, in the present study we have shown for first time, using serial measurements, that an increase in plasma adiponectin after ACS is strongly related to the risk of an adverse outcome, but that this relationship is not independent of BNP levels. Taken together with evidence of apparently paradoxical correlations of adiponectin and BNP at baseline, follow-up and in overall change following ACS, our results allude to a potential direct or indirect relationship between adiponectin and BNP post-ACS that requires further investigation.

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Appendix 5

INFORMED CONSENT FORM

You are being invited to donate a small amount of blood which will be used in the Vascular Biochemistry Laboratory, 4th Floor, Queen Elizabeth Building, Glasgow Royal Infirmary for method development or quality control purposes.

No significant risks are associated with taking blood samples. However, it can cause minor bruising and only in rare cases may cause inflammation and possible infection. The sample will be taken by a member of staff who has qualifications in phlebotomy.

Please sign below if you are willing to participate.

I agree to donate blood for the above purpose

Sign …… Date ……..

Print Name ……..

Researcher

Sign …… Date ……..

Print Name ……..